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THE 24-HOUR VARIATION IN BEHAVIOURAL RESPONSES TO 5-HT RECEPTOR STIMULATION

Submitted by P.C.MOSER B.Sc.

for the degree of

Doctor of Philosophy

of the

University of Bath, 1986

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The hours of folly are measured by the clock, of wisdom no clock can measure.

William Blake

Proverbs of Hell

CONTENTS

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SUMMARY

1. There is good evidence for more than one type of 5-hydroxytryptamine (5-HT) receptor in the CNS. Behavioural models in rats and mice thought to reflect activity at one or other of these receptors were studied over 24 hours. These were the head-twitch and 5-HT syndrome, 5-hydroxytryptophan as a discriminative stimulus, hyperactivity induced by RU 24969 and hypothermia induced by 8-OHDPAT.

2. Those thought to depend on activation of the 5-HT_2 receptor displayed a significant 24-hour variation, with the peak occurring mid light phase. Those thought to depend on activation of the 5-HT_1 receptor subtype did not show any 24-hour variation.

3. As 5-HT release varies over 24 hours, and is highest during the dark phase, it is concluded that the variation in 5-HT_2 receptor activity is driven by the rhythm of 5-HT release. 5-HT_1 receptors do not seem to adapt so readily to their degree of stimulation, and it is further concluded that they may be more likely to mediate behaviours which show a 24-hour variation in normal animals.

4. The interaction of benzodiazepines and anticholinesterases with 5-HT receptor-mediated behaviours was also studied. Benzodiazepines potentiated the head-twitches induced by direct 5-HT_2 receptor agonists but not those induced by 5-hydroxytryptophan unless the presynaptic effects of benzodiazepines were inhibited. This potentiation of 5-HT_2 mechanisms by the benzodiazepines was not mediated by the benzodiazepine receptor linked to GABA receptors, and GABA itself was shown not to be involved. The possibility of a benzodiazepine-type receptor

linked to the 5-HT_2 receptor is discussed.

5. The benzodiazepine clonazepam was found to potentiate, but not affect, the properties of the 24-hour variation in 5-HT agonist-induced head-twitches.

6. Anticholinesterases were found to inhibit the head-twitch response and RU 24969-induced hyperactivity. Both these effects seem to be manifestations of their toxicity.

1 INTRODUCTION

INTRODUCTION

Many aspects of the physiology of 5-hydroxytryptamine (5-HT) have been shown to vary over 24 hours. The most notable is that of 5-HT concentrations in the CNS, which are much higher during the light period than during the dark period. Much work has gone into explaining this variation, which is discussed in the following sections, but very little attention has been paid to variations in sensitivity of postsynaptic 5-HT receptors over 24 hours, and how this integrates with changes in presynaptic activity. One problem associated with this is the recent demonstration that multiple subtypes of the 5-HT receptor exist.

The major part of the work presented in this thesis examines the 24 hour variation in activity of behavioural models thought to indicate stimulation of one or other of these receptors. In this way it is hoped to obtain some understanding of the relationship between pre- and postsynaptic 5-HT activity and of the involvement in different 5-HT receptor subtypes in circadian rhythmicity.

Also examined was the interaction between 5-HT receptor mediated behaviours and benzodiazepines and anticholinesterases.

It is the purpose of this introduction to give readers sufficient background in the published work in these areas, and thus to allow them to evaluate the data presented in this thesis and the conclusions drawn from them.

1.1 CIRCADIAN RHYTHMS

The purpose of this section is to give a brief background to the study of circadian rhythms and their properties. A short account of what is believed to be the primary circadian oscillator, the suprachiasmatic nucleus, is also given.

1.1.1 Properties of circadian rhythms.

Circadian (from the latin : circa = about; diem = day) rhythms are just one of a range of biological rhythms that animals and plants use to regulate aspects of their physiology and behaviour, with periods ranging from milliseconds to several years. Many of these correspond to periodicities in the environment such as tidal, lunar or annual cycles and those that are designated as circadian may be defined as "an oscillation in a biochemical, physiological or behavioural function which under conditions in nature has a period of exactly 24 hours, in phase with the environmental light and darkness, but which continues to oscillate under constant but permissive conditions of light and temperature with a period of approximately but usually not exactly 24 hours" (Sweeney, 1975). The fact that these rhythms continue to be observed under constant conditions, i.e. to free-run, is generally considered to show that they are endogenously generated. However, they will synchronise, or entrain, to an external time cue as long as the period of that cue is close to 24 hours (Aschoff, 1978). The most obvious and important of these is the daily variation in light levels (Pittendrigh, 1981). This is because circadian rhythms are essentially a feature of an organism's adaptation to its environment and are thus important factors in its homeostatic

regulation of internal events and also of externally directed behaviours. Thus an animal can meet the challenge of regularly recurring events with a high degree of anticipation.

1.1.2 Neural regulation of circadian rhythms.

In mammals the generation of circadian rhythms, and the establishment and maintenance of their phase relationship to external events, is a function of the central nervous system (CNS) and recent work has indicated that these functions are localised in discrete regions of the brain. Richter (1967) placed lesions in various parts of the rat brain and found that only hypothalamic lesions affected free-running rhythms. From a series of 200 hypothalamic lesions he identified an area of the ventral hypothalamus where lesions resulted in a complete loss of circadian rhythmicity, although he did not determine the exact location responsible for their generation.

It had long been known that circadian rhythms were synchronised by environmental light-dark cycles which suggested a neural link between the retina and the rhythm generating system. However, lesions of the visual cortex or superior colliculus (Altman, 1962), of the primary and accessory optic tracts (Chase et al., 1969; Moore and Eichler, 1972; Stephan and Zucker, 1972) failed to abolish entrainment. Finally, using autoradiographic techniques Moore and Lenn (1972) and Hendrickson et al. (1972) demonstrated the presence of the long suspected pathways and found that they terminated in the suprachiasmatic nuclei (SCN) of the hypothalamus.

Subsequent to this discovery it was soon demonstrated that lesions of the SCN caused the loss of rhythmicity of adrenal

corticosterone secretion (Moore and Eichler, 1972) and of drinking and locomotor activity (Stephan and Zucker, 1972). It has since been demonstrated that the SCN are involved in the generation of circadian rhythmicity of a wide variety of physiological functions (Rusak and Zucker, 1979; Moore, 1979).

They are not, however, the only circadian pacemakers as there is good evidence for the phenomenon of internal desynchronisation in a number of species and some rhythms have been found to continue after complete lesioning of both SCN. Studies of isolated humans under constant conditions show that the free-running rhythms break up into two groups, one of which follows the rhythm of core body temperature (Aschoff, 1965; Wever, 1979; Czeisler et al., 1980). These other oscillators are not apparently located in the SCN as bilateral lesions of the SCN in monkeys, resulting in loss of rhythmicity in the sleep-wake cycle, do not eliminate the circadian rhythm of body temperature (Fuller et al., 1981; Dunn et al., 1977). The location and properties of circadian oscillators outside the SCN remain unknown and the bulk of evidence indicates that the SCN are at, or near, the top of any hierarchy of circadian pacemakers (Turek, 1985).

1.1.3 Afferent and efferent projections of the SCN.

Apart from the retino-hypothalamic pathway already described, the SCN receives inputs from a number of areas. These include the anterior hypothalamus (Conrad and Pfaff, 1976), the lateral and medial septal nuclei and nucleus of the stria terminalis, various thalamic nuclei (Pickard, 1982) and both the median and dorsal raphe nuclei (Dahlstrom and Fuxe, 1964; Saavedra et al., 1974).

Areas to which the SCN projects have been shown to include most

of those that it receives inputs from apart from the raphe nuclei, although other areas of the mid-brain are innervated, such as the interpeduncular nucleus (Sofroniew and Weindl, 1978). This probably indicates that a high level of feedback operates, but no direct evidence for this is available.

This list is not intended to be exhaustive but to indicate the range of connections made to and by the SCN and to show that it is possible for the SCN to influence functions in a variety of brain areas. A more complete list of those areas that send or receive projections to or from the SCN is available in a short review by Guldner (1985).

1.1.4 Neurochemistry of the SCN.

This is an area about which relatively little is known, but a number of substances have been detected in the SCN and a role for some of them has been suggested.

Vasopressin and neurophysin (Sofroniew and Weindl, 1980), enkephalin (Finlay et al., 1981), vasoactive intestinal peptide (VIP) (Kiss et al., 1984), corticotropin releasing factor (Palkovits et al., 1983), somatostatin and an avian pancreatic polypeptide (APP) like substance (Moore, 1983) have all been shown to exist in the SCN. There is good evidence for a role for 5-hydroxtryptamine (5-HT), which is present in the SCN (Fuxe, 1965),as is its synthesising enzyme, tryptophan hydroxylase (Brownstein et al., 1975) and its metabolising enzyme monoamine oxidase (Saavedra et al., 1976). There is also good evidence for the presence of acetylcholine (ACh), as choline acetyltransferase (Brownstein et al., 1975) and cholinergic nicotinic receptors (Segal et al., 1978) have been

localised within the SCN and they stain prominently for acetylcholinesterase (Paxinos and Watson, 1982). There is also evidence of a role for gamma-aminobutyric acid (GABA) in the SCN. High levels of GABA and its synthetic enzyme glutamic acid decarboxylase (GAD) are present in the hypothalamus of rats, monkeys and man (Perry et al., 1971). Levels of GAD are highest in interneurones of the SCN (Moore, personal communication cited by Borsook et al., 1984).

Some substances, such as vasopressin and VIP, are found primarily in cell bodies suggesting that they act as neurotransmitters either for interneurones or for efferent projections. Other substances, such as 5-HT and APP are found in nerve endings suggesting a role in afferent projections to the SCN. Kiss et al., (1984) have shown that 5-HT containing nerve endings innervate VIP neurones in the SCN.

Both acetylcholine and an APP-like neuropeptide seem to be involved in relaying information about the light-dark cycle to the SCN. Administration of carbachol mimics the phase shifting effect of light on free-running rhythms in darkness of both wheel-running activity in mice (Zatz and Herkenham, 1981) and pineal serotonin N-acetyltransferase activity in rats (Zatz, 1979), and daily administration of carbachol to hamsters can entrain the circadian rhythm of locomotor activity in a manner similar to daily light pulses. If APP is used instead, it mimics the phase shifting effects of dark pulses on free-running rhythms in constant light of wheelrunning activity (Albers et al., 1984). These two substances thus appear to play a role in the entrainment of the circadian system by the light-dark cycle. The phase delaying effects of light pulses in the hamster can be prevented by the GABA

receptor antagonist bicuculline (Ralph and Menaker, 1983), which suggests that GABA may also play a role in entrainment.

A number of studies using iontophoretic administration of drugs to the SCN have been reported. The most interesting involve two substances thought to act as neurotransmitters in the SCN, namely 5-HT and ACh. Direct application of 5-HT to the SCN, and stimulation of the dorsal raphe nucleus both act to inhibit firing of the SCN (Groos et al., 1983) while ACh increases the firing rate of SCN neurones (Nishino and Koizumi, 1977). The cells that respond to ACh also, to a large extent, respond to optic nerve stimulation, an observation that ties in well with the phase shifting action of carbachol that mimics light pulses. It has also been shown that GABA can inhibit spontaneous firing of neurones in slices of rat hypothalamus (Borsook et al., 1984).

The observed effect of 5-HT suggests that this neurotransmitter may also play a part in the modulation of pacemaker function. Martin and Marsden (1985) have shown that 5-HT turnover in the SCN, as measured by in vivo voltammetry and intracerebral dialysis, is highest during the dark phase when the firing rate of the SCN is lowest (Inoye and Kawamura, 1980) which would support such a role. However depletion of 5-HT does not affect the circadian period (Honma et al., 1979) which suggests it may not be a primary modulator.

1.2 CIRCADIAN RHYTHMS IN PARAMETERS OF CNS 5-HT FUNCTION

This area of research has generated a considerable number of reports, and it is beyond the scope of this section to cover it in detail. The following sections give brief summaries of the work that has been done, but is by no means exhaustive.

1.2.1 Circadian variation in 5-HT concentrations

A circadian rhythm in the concentration of 5-HT in the CNS of rodents is now well established and has been reported by many authors (e.g. Morgan et al., 1976; Hillier and Redfern, 1977) since it was first demonstrated in the mouse by Albrecht et al., (1956). It has also been shown to exist in cats (Reis et al., 1969) and ferrets (Yates and Herbert, 1979). The peak of this rhythm in adult animals is found in the light phase, although ontological variations exist (Okada, 1971). It has also been shown that the amplitude of this rhythm is not the same in all brain regions. Quay (1968) reported that the highest amplitudes are found in the frontal cortex, the hypothalamus and the lower brainstem. More recently it has become possible to examine this rhythm in individual brain nuclei, and data reported by Martin and Marsden (1985) concerning the circadian variation in 5-HT content of the SCN shows that there is a marked rhythm, the 5-HT concentration varying from about 7 pg.µg protein^{-1} at mid-dark to over 14 pg.µg protein^{-1} at mid-light.

Much effort has been applied to understanding the reasons for this variation in 5-HT levels and the following section briefly reviews some of the work that has been done in this area.

1.2.2 Precursor availability

The initial step in the synthesis of 5-HT involves the hydroxylation of its precursor amino acid, L-tryptophan (TRY) to 5-Hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (Udenfriend, 1959). The affinity of this enzyme for its substrate is low, and it has been suggested that it may not be saturated by usual concentrations of TRY in neurones (Lovenberg et al., 1964). Experiments by Fernstrom and Wurtman (1971) showed that very small changes in plasma and brain TRY, of a similar magnitude to those that occur naturally, could significantly elevate brain 5-HT concentrations. As a number of reports indicate a circadian variation in the availability of TRY in both plasma and brain (Rapoport et al., 1966; Hery et al., 1977b; Martin and Redfern, 1982) it is possible that the changes in 5-HT concentrations over 24 hours occur as a direct result of changes in tryptophan availability. However, levels of TRY in the plasma and brain are highest during the dark phase (Fernstrom and Wurtman, 1971; Hillier, 1974) and are thus 180^{o} out of phase with the rhythm of 5-HT concentrations, which are highest during the light phase. As the results of Fernstrom and Wurtman (1971) showed rapid effects of increasing peripheral levels of TRY on brain 5-HT levels, it would appear that precursor availability is not the primary reason for the changes in 5-HT concentration over 24 hours, although it can obviously have an affect. Hutson et al. (1984) have recently measured CSF concentrations of TRY using in vivo dialysis, and find that there is no significant change over 24 hours. This result also suggests that the availability of TRY is not the cause of the variation in 5-HT concentration over 24 hours.

1.2.3 Tryptophan hydroxylase activity

Tryptophan hydroxylase is the rate limiting enzyme in the synthesis of 5-HT (Ashcroft et al., 1965), and it has consequently been examined in some detail as a possible source of the diurnal variation in brain 5-HT concentrations. However, the results obtained by various groups have been conflicting. In 1977, Kan et al. reported that the activity of tryptophan hydroxylase exhibited a significant circadian variation in the rat, and similar findings have also been reported by Cahill and Ehret (1981), again in the rat, and by Natali et al. (1980) in the mouse. The peak of these circadian rhythms was found to occur in the dark period, i.e. out of phase with the peak in 5-HT levels.

Other workers however, have failed to show a rhythm in the activity of this enzyme using the same techniques, where the activity of the enzyme is measured in the supernatant fraction of homogenised brain (Deguchi, 1977; McLennan and Lees, 1978). Using a synaptosomal preparation Brown et al. (1982) also failed to demonstrate a significant circadian rhythm in tryptophan hydroxylase activity.

1.2.4 5-hydroxytryptophan decarboxylase activity

Following the conversion of TRY to 5-HTP, the final step in the synthesis of 5-HT is the decarboxylation of 5-HTP by the enzyme 5-HTP decarboxylase. This enzyme is present in monoamine neurones and is highly active, being involved in the synthesis of both noradrenaline and dopamine, as well as 5-HT (Anden et al., 1965). There is some evidence that different enzymes are involved in the decarboxylation of 5-HTP and L-dihydroxyphenylalanine (DOPA), the precursor of dopamine,(Clark et al., 1954), but if this is so,

both are capable of metabolising 5-HTP and DOPA. Hokfelt et al. (1973) could only find one enzyme in samples of rat brain tissue, and this was capable of decarboxylating both substrates. Yuwiler et al. (1960) have provided evidence that DOPA and 5-HTP compete for the same enzymatic site and that the enzyme shows a preference for the decarboxylation of DOPA.

Hillier and Redfern (1976) found that although the rate of 5-hydroxytryptophan decarboxylase activity in crude brain homogenate varied significantly over 24 hours, the activity of the purified enzyme itself did not. They concluded that the variation that was observed in brain homogenate was due to other factors, such as substrate competition or cofactor availability. They also demonstrated that the enzyme is normally unsaturated,which would make it unlikely that it contributes to the rhythm of 5-HT over 24 hours.

1.2.5 5-HT release

In considering the importance of TRY availability or metabolism, an underlying assumption seems to be that the changes in 5-HT levels are of functional significance. However, data from a variety of sources indicates that functional activity of serotonergic neurones is highest during the dark phase, i.e. the period when 5-HT levels are lowest, which suggests that 5-HT concentrations do not play a major role in controlling 5-HT release into the synapse.

Measurement of extracellular 5-hydroxyindole acetic acid (5-HIAA), the major metabolite of 5-HT, over 24 hours by either intracerebral dialysis (Martin and Marsden, 1985), in vivo voltametry (Faradji et al., 1984) or by repeated sampling of cerebrospinal

fluid (CSF) (Hutson et al., 1984) reveals that levels are significantly higher during the dark phase than they are during the light phase. This strongly indicates that the amount of 5-HT released is also higher during the dark phase. Measurement of 5-HT concentrations in the brain using either intracerebral dialysis or repeated CSF sampling does indeed show that the amount of 5-HT being released is highest during the dark phase, unlike the tissue levels of 5-HT which are highest during the light phase.

1.2.6 5-HT uptake

This aspect of 5-HT function has been little studied over 24 hours, but the available data shows that it also exhibits a circadian variation. Meyer and Quay (1976) measured 5-HT uptake in vitro in the SCN and found a significant circadian variation, with highest activity during the dark phase and lowest activity during the light phase. Wirz-Justice et al. (1983) also found that imipramine binding in the SCN followed a similar rhythm. It has been suggested that imipramine binding is related to 5-HT uptake (Briley, 1985).

This rhythm is thus in phase with that for 5-HT release, which is as expected, as re-uptake is the primary means of terminating the transmiter action of 5-HT, which would need to be at its most active when 5-HT neurones themselves are most active.

1.2.7 Are 5-HT levels of functional importance ?

While many of the factors that affect the biosynthesis of 5-HT show a circadian variation, as discussed in this section, the relationship between these variations and the well established rhythm in 5-HT levels remains unclear. Very few of the steps seem

to have characteristics that would make them reliable regulators of neurotransmitter function. Levels of tryptophan in the CNS can be markedly affected by diet (Fernstrom, 1979) and the activity of tryptophan hydroxylase is 180° out of phase with 5-HT levels, and although this may reflect a lag phase it would not allow rapid adaptation to functional requirements. The activity of 5-hydroxytryptophan decarboxylase appears to be open to a variety of influences that may be part of a control mechanism for 5-HT function but the fact that this enzyme is normally unsaturated would indicate that it too is not of controlling importance. Of course, it is always possible that the function of 5-HT in the CNS is that of a general modulator, and that precise control of its activity is not desirable. All the factors mentioned above may thus be involved in shaping the overall activity of 5-HT. On the other hand it does not seem reasonable that changes in the functional activity of 5-HT neurones are as a result of changes in 5-HT concentration. It seems more likely that the observed changes in presynaptic functions are designed to replenish stores of transmitter that are used at various rates over 24 hours.

It therefore seems likely that the recently determined changes in 5-HT outflow are at least partly responsible for the observed daily fluctuations in 5-HT levels. However, the fact that the re-uptake of 5-HT is also highest when outflow is highest, may mean that the effect of 5-HT release on 5-HT concentrations is minimal.

What is perhaps most important is the observation that presynaptic activity is highest during the dark phase. The next step in the functional pathway of 5-HT activity is an action upon a postsynaptic receptor, and determining how the activity of these

change over 24 hours is the primary aim of this thesis. In this way it is hoped that an overall picture can be obtained of how 5-HT functional activity changes over 24 hours. However, the postsynaptic 5-HT receptor population does not appear to be homogeneous and the following section discusses the evidence that has accrued over the last few years for multiple subtypes of the 5-HT receptor.

1.3 MULTIPLE 5-HT RECEPTORS

5-HT is now well established as a neurotransmitter, both in the periphery and in the CNS of mammals. All of the criteria that are considered essential for a transmitter role have now been fulfilled: systems for its synthesis (see Section 1.2), storage, release and for its inactivation have all been described (Van Praag, 1970). Also, as already described in Section 1.1, application of 5-HT by microiontophoresis to the SCN mimics electrical stimulation of the raphe nuclei (Groos et al., 1983). 5-HT is widely distributed in the CNS and its pathways have been well mapped since the method of fluorescence histochemistry was adapted for this use by Falck et al. (1962). Using this technique it was found that nearly all the 5-HT containing cell bodies were restricted to the raphe nuclei, which lie in the mid-portion of the lower pons and upper brain stem (Fuxe, 1965). From here there are pathways to most regions of the CNS (Anden et al., 1966; Dahlstrom and Fuxe, 1964).

Consistent with this wide distribution of 5-HT terminals is the fact that 5-HT appears to be involved in a wide range of behaviours, disease states and drug actions. These include sleep, eating, sexual activity, release of pituitary hormones, perception of pain, depression, schizophrenia and the action of hallucinogens (Barchas and Usdin, 1973). This wide variety of actions made it difficult to study specific effects of 5-HT in the CNS, and the lack of speciric pharmacological agents compounded this difficulty. However, the recent evidence for subtypes of the 5-HT receptor and the development of drugs with a degree of specificity for these subtypes has rejuvenated the study of 5-HT and published work in this field has increased rapidly over the last few years.

It was through the differential activities of drugs in the periphery that evidence for multiple 5-HT receptors first appeared. In 1954, Gaddum and Hameed showed that it was possible to block the 5-HT response in the rabbit ear and rat uterus with LSD, but not in the guinea pig ileum. These findings were subsequently confirmed by a number of studies and this area has been reviewed by Wallis (1981). As evidence accrued for a transmitter role for 5-HT in the CNS, the presence of a heterogeneous population of receptors was used to explain a number of results.

There is now good evidence to suggest that there are at least two, and possibly three, subtypes of the 5-HT receptor. The evidence for this comes from electrophysiological, ligand binding, biochemical and behavioural studies. The first three will be reviewed in this section, while the behavioural data will be discussed in Section 1.4.

1.3.1 Electrophysiological evidence for multiple 5-HT receptors

One of the first techniques used to study central 5-HT actions directly was that of microiontophoresis. When 5-HT was administered in this way, Bloom et al. (1972) found that its primary action was that of inhibiting neuronal activity in almost all areas of the brain studied, but it was also found to have excitatory actions in a few areas. This study also demonstrated that the predominant outcome of raphe cell stimulation was inhibitory. This work was extended by Haigler and Aghajanian (1974, 1977) whose work indicated the presence of presynaptic receptors and which also showed that the excitatory effects of 5-HT could be antagonised by the classical 5-HT antagonists such as cyproheptidine and methysergide, but that

the inhibitory actions were unaffected. The pharmacology of the presynaptic receptor, which decreased firing rates of 5-HT neurones, was found to be different to that of the postsynaptic receptors, and Aghajanian and Haigler (1975) showed that the more drugs could discriminate between pre- and postsynaptic sites the more hallucinogenic they became (e.g. LSD is three times more potent as an inhibitor of 5-HT neuronal activity than against postsynaptic receptors).

Aghajanian (1981) has published a classification of 5-HT receptors based on results from electrophysiological studies which he has designated S_1, S_2 and S_3. Activation of the S_1 receptor facilitates the depolarising action of excitatory amino acids and it is readily blocked by the classical 5-HT antagonists. The S_2 receptor seems to be localised to the cell bodies of 5-HT neurones and inhibits the activity of these cells. This receptor is particularly sensitive to hallucinogenic agents such as LSD. The third subtype, S_3, reduces the firing rates of neurones but is not antagonised by classical 5-HT antagonists, and LSD is only weakly active.

In contrast to some of these results are those of Jones (1982), who showed that methergoline could inhibit the depressant effect of 5-HT. In these experiments the 5-HT effect was induced by stimulation of the raphe nucleus rather than by application of exogenous 5-HT as in previous experiments. Jones (1982) has also suggested that the inhibitory responses are mediated by tryptamine rather than 5-HT.

1.3.2 Ligand binding studies

The first radioactive ligands used to study 5-HT receptors were [^{3}H]-5-HT and [^{3}H]-LSD. Both of these ligands exhibited high affinity for sites on brain membranes and saturability, and both ligands displayed properties consistent with their binding to 5-HT receptors. 5-HT, 5-HT agonists and antagonists more potently displaced both ligands than agents associated with other neurotransmitter systems (Bennett and Aghajanian, 1974; Bennett and Snyder, 1975, 1976; Lovell and Freedman, 1976). It was immediately obvious that there were marked differences in the properties of these two binding sites. Indole ring containing agonists had a much higher affinity for the [^{3}H]-5-HT site, wnereas antagonists had a much greater affinity for the [^{3}H]-LSD sites. This was originally interpreted as the binding of ligands to agonist and antagonist states of the same receptor. Leysen et al. (1978) then showed that the neuroleptic [^{3}H]-spiperone, which labels dopamine receptors in the striatum, labelled sites in the frontal cortex which displayed characteristics of a 5-HT receptor. 5-HT had a far higher affinity for this site than did dopamine, and the potency of a variety of agents to displace [^{3}H]-spiperone correlated with their ability to antagonise tryptamine induced seizures in the rat.

The first suggestion that distinct binding sites were involved came from Peroutka and Snyder (1979). They showed that [^{3}H]-5-HT and [^{3}H]-spiperone labelled separate sites and that [^{3}H]-LSD bound to them both. They designated the [^{3}H]-5-HT site the 5-HT$_1$ receptor and the [^{3}H]-spiperone site the 5-HT$_2$ receptor.

Leysen et al. (1978) had already shown that the potency of a group of compounds for inhibiting [^{3}H]-spiperone binding correlated

with their ability to antagonise tryptamine seizures and in 1981, Peroutka et al. demonstrated that this binding also correlated with potency for inhibition of 5-HTP-induced head-twitches in mice. In contrast there was no correlation between affinities for the [^{3}H]-5-HT site and inhibition of head-twitches. It therefore seemed likely that the 5-HT_2 site represented a functional 5-HT receptor, and a large body of evidence now supports the mediation of the head-twitch response and the discriminative stimulus properties of hallucinogens by this receptor (see Section 1.4). A more selective ligand for the 5-HT_2 site was introduced in 1981 by Leysen et al. who showed that [^{3}H]-ketanserin would label the same sites as [^{3}H]-spiperone, but did not bind to dopamine receptors (Leysen et al., 1982). This enabled a wider study of 5-HT_2 binding sites in the brain.

The 5-HT_1 site seems to be a heterogenous population of sites. Bennett and Snyder (1976) had already noticed that the order of potency of some compounds for inhibiting [^{3}H]-5-HT binding varied between brain regions and Nelson et al. (1978) found that the binding of some compounds was consistent with multiple binding sites. When spiperone was used to displace [^{3}H]-5-HT it was found to produce a distinctly biphasic inhibition curve (Pedigo et al., 1981). These sites were designated 5-HT_{1A} and 5-HT_{1B} for those having high and low affinity for spiperone respectively. Further evidence that these represented distinct sites came with the use of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OHDPAT). This compound showed high affinity to 5-HT_{1A} sites only (Middlemiss and Fozard, 1983). When given to rats this compound induces signs of the 5-HT syndrome (Hjorth et al., 1982) indicating that it is a 5-HT agonist (Jacobs,

1976) but these effects are not antagonisable with classical 5-HT antagonists following administration of reserpine (Tricklebank et al., 1985). However, the β-adrenoceptor antagonists pindolol and propranolol will antagonise these effects and this antagonism is found to be stereoselective, with only the (-)-isomers proving effective. Nahorski and Willcocks had previously (1983) shown that some β-adrenoceptor antagonists could selectively displace ligands at the 5-HT_1 site, and more recent binding data shows that the (-)-isomers of these compounds are potent displacers of ligands for the 5-HT_{1A} binding site (Middlemiss, 1984; Middlemiss et al., 1985). The (+)-isomers show much less selectivity for subtypes of the 5-HT receptor.

The regional distribution of the various binding sites provides further evidence that they are distinct sites, rather than different states of the same receptor. 5-HT_1 binding sites show highest levels in the hippocampus and striatum, whereas 5-HT_2 binding sites are most prominent in the prefrontal cortex, tubercular olfactorium, nucleus accumbens and striatum. Neither of these distributions corresponds to the distribution of 5-HT uptake, nor 5-HT levels (Leysen et al., 1985).

1.3.3 Functional correlates of 5-HT binding sites

The relative ease of ligand binding studies has meant that this system of classification has become the most widely used, and many supposed roles for various receptors has rested on correlations between drug potencies in functional tests and binding affinities. This has led to many anomalies, and none of the proposed roles for the 5-HT_1 site are without conflict. Peroutka et al., (1981)

originally proposed that the 5-HT_1 site was linked to adenylate cyclase. However, this is refuted by the data of Nelson et al. (1980) showing that 5-HT stimulated adenylate cyclase and 5-HT_1 binding sites have different regional and subcellular distributions, and Leysen and Tollenaere (1982) have found no correlation in the abilities of compounds to interact with either site.

Another proposed role for the 5-HT_1 binding site is that of an autoreceptor function (Martin and Sanders-Bush, 1982; Engel et al., 1983). There are many discrepancies between these two studies concerning relative potencies of drugs, and in their reviews of this area both Leysen (1985) and Moret (1985) concluded that the evidence for an autoreceptor role for the 5-HT_1 binding site was far from convincing. The emergence of the possibility of subtypes of the 5-HT_1 receptor makes previous work difficult to evaluate, but the recent use of 8-OHDPAT, which exhibits selectivity for the 5-HT_{1A} site, has provided some interesting data. Unlike [^{3}H]-5-HT binding sites, the number of [^{3}H]-8-OHDPAT binding sites is reduced by destruction of 5-HT neurones (Gozlan et al., 1983) which strongly indicates a presynaptic location, although potency to displace [^{3}H]-8-OHDPAT by a variety of compounds does not correlate with their activity in the release studies of Martin and Sanders-Bush (1982) and Engel et al. (1983). A clue to the position of the [^{3}H]-8-OHDPAT binding site may come from the electrophysiological studies carried out by Fallon et al. (1983) who found that 8-OHDPAT readily depressed the firing rate of 5-HT neurones when given systemically, and by de Montigney et al. (1984) who found it to be as potent as LSD when directly applied to dorsal raphe neurones. This suggests that 8-OHDPAT can act at the receptor classified as S_2 by Aghajanian

(1981). The exact relationship between this receptor and the 5-HT_{1A} site is unclear but the autoreceptor described by Cerrito and Raiteri (1979) shows much in common with the S_2 site as LSD is a potent agonist in both systems and methiothepin acts as an antagonist in both.

However, overall there is little congruence between the two systems of classification. The reasons for this probably lie in the lack of suitable agonists and antagonists for many of the 5-HT receptors, and also for discrepancies in the binding data from different groups. Some of the problems involved in the use of ligand binding have been discussed by Leysen (1984) and by Schnellman et al. (1984) who highlight the species differences observed in binding characteristics.

Finding a functional correlate for the 5-HT_2 site has proved a lot easier, and there is far more general agreement about these measures than for those used for the 5-HT_1 site. Most of these have been behavioural in nature, and these will be discussed in the next section. One that is biochemical, however, is 5-HT stimulated inositol phospholipid breakdown in brain slices. Both Kendall and Nahorski (1984) and Conn and Sanders-Bush (1984) have provided evidence that this is mediated by a 5-HT_2 receptor, and suggest that this is the biochemical effector system linked to these receptors. The 5-HT_2 antagonist ketanserin blocked the response, but (-)-pindolol would not, and down regulation of 5-HT_2 receptors was accompanied by a loss of responsiveness of inositol phospholipid metabolism to 5-HT. Godfrey et al. (1985) have also reported an RU 24969-stimulated inositol phospholipid breakdown which was not antagonised by either ketanserin or (-)-propranolol. Iprindole and

amitriptyline did antagonise the response however, which suggests similarities with the 5-HT receptor described by Quach et al. (1982). They described a 5-HT-stimulated glycogenolysis which did not show any correlation with potencies against 5-HT_1 or 5-HT_2 binding sites but which was antagonised by antidepressants.

There is clearly a lot more work required to characterise the 5-HT receptors involved in some of the responses discussed above. The currently popular system of classification based on data obtained using the ligand binding technique, and used in this thesis, may need to be reassesed despite being useful and rapid for the evaluation of new compounds. Of all the problems that this classification has, the most fundamental is the apparently irreconcilable differences it has with the electrophysiological classification. This latter system is at least based on functional effects, an area which is proving to be a prime drawback in accepting the 5-HT_1 site as a receptor.

A number of recent studies have explored the effects of drugs developed from ligand binding techniques using electrophysiological methods. Lakoski and Aghajanian (1985) have studied the effects of ketanserin on the inhibitory responses to microiontophoretically applied 5-HT in the prefrontal cortex, lateral geniculate and dorsal raphe nucleus. In the raphe nucleus ketanserin had no effect alone, or on the responses to 5-HT, but in the other brain areas studied ketanserin potentiated the inhibitory effects of 5-HT. Davies et al. (1985) were able to examine the effects of ketanserin on both inhibitory and excitatory actions of 5-HT in the rat brainstem. In agreement with Lakoski and Aghajanian (1985), no antagonism by ketanserin was observed on the inhibitory effect of 5-HT on neuronal

firing in this brain area, but they did find that it would antagonise the 5-HT-induced neuronal excitation when given peripherally. Essentially the same results were obtained with methysergide, which shows far less selectivity for the 5-HT_2 site (Leysen et al., 1981), but Davies et al. (1985) suggest that they did not use a high enough dose to rule out the involvement of 5-HT_1 receptors.

Studies of this type are needed to bring the ligand binding data, the electrophysiological data and the animal model data together into a coherent system. The studies discussed above, and those with 8-OHDPAT discussed earlier, clearly point to parallels between the S_1 and 5-HT_2 receptors, and between the S_2 and 5-HT_{1A} receptors, but there are still large differences, such as the ability of LSD to bind to all 5-HT sites but to have an action at only one of the electrophysiologically-characterised sites.

1.4 BEHAVIOURAL MEASURES OF 5-HT RECEPTOR ACTIVITY

Unlike some other neurotransmitter systems, the study of CNS serotonergic activity is well blessed with whole animal models. Some of these, such as the head-twitch response, are very well established, and can be convincingly shown to result from increased activity of the serotonergic system. Other, more recent, models are considerably less well established and can only be tentatively ascribed to this system.

With the recent explosion of interest in 5-HT, this particular area has been extensively reviewed in recent years and for a more extensive overview of these models, the reader is directed to Peroutka (1984), Leysen et al. (1984), Green (1984), Green and Heal (1985) and Tricklebank (1985).

1.4.1 The head-twitch response

This model was first described in mice by Corne et al. (1963) and is based on the observation that injection of 5-HTP, the precursor of 5-HT, produced a characteristic head-twitch behaviour which closely resembled the pinna-reflex in appearance. The response correlated with brain stem levels of 5-HT and was potentiated by monoamine oxidase inhibitors. Other 5-HT agonists were also shown to induce head-twitches in mice, including mescaline and LSD (Corne and Pickering, 1967), quipazine (Malick et al, 1977) and 5-methoxy-N,N-dimethyltryptamine (5-MeODMT) (Friedman and Dallob, 1979).

The response has also been reported in the rat where it has been described as 'wet dog shake' (Bedard and Pycock, 1977; Matthews and Smith, 1980). As in mice, it is induced by a range of 5-HT agonists and appears to be mediated via serotonergic systems in the

hindbrain (Bedard and Pycock, 1977; Przegalinski et al., 1977). Direct application of 5-HT into the CNS also elicits head-twitches (Drust and Connor, 1983).

In both species this behaviour is inhibited by all the so-called 'classical' 5-HT antagonists and more recent studies with the selective 5-HT_2 antagonists pirenperone and ketanserin (Yap and Taylor, 1983; Green et al., 1983; Colpaert and Janssen, 1983b) demonstrate that they too are potent antagonists of this response. These authors have concluded that the head-twitch response is brought about by activation of the 5-HT_2 receptor subtype, in confirmation of the suggestion by Peroutka et al. (1981). This is further reinforced by the results of Heal et al. (1985) showing that increases in 5-HT_2 receptor number and head-twitch response are correlated following destruction of presynaptic 5-HT neurones. Middlemiss (1982), however, was unable to find a correlation between ability to displace [^{3}H]-spiperone binding and inhibition of head-twitches. One possible explanation for this is that other actions of the drugs he used were modifying either the binding of the drug or the head-twitch response, as many other neurotransmitter systems have been shown to affect the head-twitch, as detailed below.

There is considerable evidence that noradrenaline can influence brain serotonergic activity (see Green and Heal, 1985) as α_2-adrenoceptor agonists will inhibit the head-twitch response in mice, while α_2-adrenoceptor antagonists potentiate the response (Handley and Brown, 1982). The same workers also suggest that α_1-adrenoceptor agonists potentiate the head-twitches. Inhibition of β-receptors seems to have no effect on head-twitches, but β-agonists were found to potentiate the response (Handley and Singh, 1984).

It has also been reported that benzodiazepines will affect the head-twitch response. This will be discussed in section 1.5.

There seems little doubt, however, that the initiating receptors are of the 5-HT_2 subtype, particularly as neither of the recently introduced 5-HT_1 agonists RU 24969 and 8-OHDPAT have been found to induce head-twitches (Green et al., 1984; Green, 1984). Other compounds such as thyrotropin releasing hormone and enkephalins can induce head-twitches but the mechanisms seem to be totally separate to those that mediate the 5-HT-agonist induced head-twitch (Drust and Connor, 1983).

1.4.2 The 5-HT syndrome

The group of behaviours that make up the 5-HT syndrome have been suggested to reflect a "pure behavioural index of central serotonergic activity" (Jacobs and Klemfuss, 1975). This is not accepted quite so readily nowadays, but the syndrome has continued to be of use in the study of central 5-HT activity and mechanisms.

The syndrome consists of hyper-reactivity, hyperactivity, resting tremor, rigidity, straub tail, reciprocal fore-paw treading, hindlimb abduction and lateral head-weaving. In the early work only the hyperactivity component was studied to any great extent (Grahame-Smith, 1971a,1971b; Modigh, 1972) but a review of the whole syndrome as an animal model for studying CNS 5-HT was given by Jacobs (1976), who details the evidence for its mediation by a 5-HT receptor. Administration of a variety of 5-HT agonists will produce the syndrome and it is unaffected by catecholamine synthesis inhibition (Jacobs, 1974). Compounds which release 5-HT, such as p-chloro-amphetamine and fenfluramine also induce the syndrome (Trulson and

Jacobs, 1976) and inhibition of 5-HT uptake potentiates the syndrome induced by 5-HTP (Ortmann et al., 1980). Like the head-twitch, the 5-HT syndrome is mediated by brain stem mechanisms (Jacobs and Klemfuss, 1975).

The early quantification of the behaviours involved in the syndrome assumed that a single mechanism was responsible for each component, and the scoring methods used reflected this. However, Dickinson et al. (1983) have recently reported that the scoring method used, ranging from all-or-nothing to a graded rating of each component, can have profound effects on the conclusions that may be drawn from the results. There is now considerable evidence that the 5-HT syndrome is a complex manifestation of central 5-HT mechanisms. When, for example, it was induced by quipazine, it was antagonised by cyproheptidine, cinanserin and mianserin. However these drugs were inneffective against apparently the same syndrome induced by 5-MeODMT (Green et al., 1981). Methysergide, methergoline and (-)-propranolol were capable of blocking the syndrome induced by both agonists. Green et al. (1981) rationalised these results with the concept of strong and weak agonists and antagonists of the 5-HT receptor. There were also some indications in the data that the different components of the syndrome responded differently to the antagonists, with hind-limb abduction being more resistant to antagonism than head-weaving and fore-paw treading. Also observed were differences in the effect of the antagonists on the animals reactivity to a sound stimulus. This strongly indicates that different mechanisms are involved in each of the components and this view is further reinforced with the results of recent experiments using 8-OHDPAT. This compound shows high affinity for the 5-HT_{1A}

site in ligand binding studies which is several orders of magnitude greater than its affinity for the 5-HT_{1B} or 5-HT_2 site (Middlemiss and Fozard, 1983). In normal rats this compound will induce many of the components of the 5-HT syndrome, which can be antagonised not only by 5-HT_{1A} antagonists such as (-)-pindolol, but also by the 5-HT_2 antagonists ketanserin and pirenperone and by the α_1-adrenoceptor antagonist prazosin (Tricklebank et al., 1985). Lucki et al. (1984) have reported that neither ketanserin nor pirenperone would antagonise the syndrome induced by 5-MeODMT and concluded that the 5-HT_1 receptor was responsible.It is difficult from these results to accurately define the role of different 5-HT receptors in the 5-HT syndrome response. There is some evidence that it is the α-adrenoceptor antagonist properties of ketanserin that are responsible ror the inhibition of the 5-HT syndrome induced by 8-OHDPAT, as ritanserin, a recently introduced and more selective 5-HT_2 antagonist, does not inhibit the 8-OHDPAT induced effects. Ritanserin does inhibit the 5-HT syndrome when it is induced by quipazine (Goodwin and Green, 1985).

Clear evidence for the role of catecholaminergic neurones in some aspects of the syndrome, and also for different pathways mediating different components of the syndrome comes from the work of Tricklebank et al. (1985). The properties of the 5-HT syndrome induced by 8-OHDPAT in normal rats have been mentioned above, but if 8-OHDPAT is given to reserpinised animals these properties differ markedly. In rats pretreated with reserpine, which depletes stores of monoamines, 8-OHDPAT did not induce head-weaving or hyperactivity but fore-paw treading remained unaffected and the flat body posture was enhanced. The response of these remaining behaviours to

antagonists was also found to have changed. After reserpine, only 5-HT_{1A} antagonists inhibited the fore-paw treading. The 5-MeODMT-induced syndrome behaves in a similar way after reserpine pretreatment (Tricklebank, 1985).

The simplest explanation for these findings is that a 5-HT_{1A} receptor link exists between the 5-HT_2 receptors and the behavioural response (Goodwin and Green, 1985). The results of Tricklbank et al. (1985) also show that catecholaminergic neurones are heavily involved in the manifestation of the syndrome.

There is thus good evidence for a role of both 5-HT_{1A} and 5-HT_2 receptors in the mediation of the 5-HT syndrome, which decreases its usefulness as an analytical tool for studying 5-HT mechanisms. Another point raised by Goodwin and Green (1985) is the difficulty in translating results from one species to another, as they found that 8-OHDPAT did not induce the 5-HT syndrome in mice. They also found that (-)-propranolol had different selectivities in rats and mice. Jones and Dourish (1982) have previously shown that there are profound strain differences between Sprague-Dawley and Wistar rats as regards responses to 5-HT agonists, and it is not therefore surprising that the differences between rats and mice should be at least as marked.

1.4.3 5-HT agonist-induced hyperactivity

Increased locomotor activity has often been included as part of the 5-HT syndrome (Jacobs, 1976), but it has become apparent that it is separate from the other components. Green et al. (1981) demonstrated that while methysergide and methergoline antagonised the head-weaving, fore-paw treading and hindlimb abduction induced by

5-MeODMT, the locomotor activity component was enhanced. Deakin and Green (1978) had also shown that (-)-propranolol would inhibit all these behaviours. This is consistent with the locomotor activity component being mediated by a different mechanism, and as (-)-propranolol is a potent antagonist of the 5-HT_1 receptor (Nahorski and Willcocks, 1983), would imply that the 5-HT_1 receptor is involved in this aspect of 5-MeODMT induced behaviours.

This view was reinforced with the introduction of RU 24969, a compound which potently displaces binding at the 5-HT_1 site (Hunt and Oberlander, 1981) and is slightly more selective for the 5-HT_{1B} site than for the 5-HT_{1A} site (Tricklebank, 1985; Cortes et al., 1984). When given to rats or mice this compound will induce a marked increase in locomotor activity. In rats this occurs in bursts and resembles that seen in rats given 5-MeODMT after 5-HT_2 receptor inhibition (Green et al., 1984; Gardner and Guy, 1983; Green and Heal, 1985).

However, there is some doubt that the response to RU 24969 is primarily based on 5-HT receptor stimulation. Pretreatment with methysergide or methergoline actually enhances the hyperactivity in rats, which suggests a 5-HT_2 receptor-mediated inhibitory component (Green and Heal, 1984). However, the same authors found that the more selective 5-HT_2 receptor antagonist, pirenperone, inhibited the response. This antagonism may be due to pirenperone's properties at the dopamine receptor where it is as potent as haloperidol (Green et al., 1983). Green et al. (1984) and Oberlander and Boissier (1981) both report that haloperidol will inhibit the response in rats, and Green et al. (1984) also found that (-)-propranolol would not antagonise the RU 24969-induced hyperactivity.

As (-)-propranolol is a reasonable antagonist at 5-HT_1 receptors (Nahorski and Willcocks, 1983), this is not consistent with an involvement of the 5-HT_1 receptor in this response. In contrast, Tricklebank (1984a) reported that propranolol can inhibit the response to RU 24969 in the rat, although non-stereoselectively, which again suggests that the 5-HT_1 receptor is not involved. Green and Heal (1985) have suggested that RU 24969 is acting at a 5-HT receptor at which (-)-propranolol is not an antagonist, as propranolol does not affect the decrease in 5-HT synthesis induced by RU 24969 (Green et al., 1984). In support of this, Middlemiss (1984) has provided evidence that (-)-propranolol shows some selectivity as an antagonist of the 5-HT_{1A} receptor.

The response in mice to RU 24969 is similar to that of rats, but a more continuous hyperactivity is observed which is weakly antagonised by methergoline and propranolol (Gardner and Guy, 1983; Green et al., 1984).

Hyperlocomotion is also induced in rats, but not in mice, by the 5-HT_{1A} agonist 8-OHDPAT (Tricklebank et al., 1984). This effect is reversed by haloperidol and reserpine administration, but is not blocked by pindolol and is actually enhanced by propranolol (Tricklebank et al., 1984).

Clearly the evidence for hyperlocomotion induced by 5-MeODMT, RU 24969 and 8-OHDPAT being mediated by a 5-HT receptor is equivocal and proof will require the development of selective antagonists. It is clear, however, that catecholaminergic pathways are involved in the response.

An animal model recently described by Blackburn et al. (1984a, 1984b) may shed some light on this situation and provide evidence

that a 5-HT receptor is involved in the response to RU 24969. Rats were prepared with unilateral 5,7-DHT lesions of the dorsal raphe nucleus which resulted in an increase in the number of 5-HT_1 binding sites in the substantia nigra on the lesioned side. Administration of non-selective 5-HT agonists such as 5-MeODMT caused a dose related contralateral rotation, which was not antagonised by the 5-HT_2 antagonists pirenperone and ketanserin. It was also found that RU 24969 and 8-OHDPAT, but not quipazine, would cause a contralateral rotation. These results rule out the involvement of the 5-HT_2 receptors but give little direct evidence for the subtype of 5-HT receptor involved, other than that a 5-HT_1 subtype seems to be responsible. Once again, better antagonists are required to classify this behaviour, but the results of Blackburn et al. (1984a, 1984b) indicate that 5-HT receptors can be involved in increased locomotor activity.

1.4.4 Other models

When administered to guinea-pigs, 5-HTP will induce a dose-related myoclonus (Klawans et al., 1973). This model has subsequently been examined in more detail by Luscombe et al. (1981, 1982) and by Jenner et al. (1984) who suggest that this behaviour is mediated by 5-HT_1 receptors in guinea-pig brainstem, although their data is by no means conclusive.

Another model that has been widely used as a tool for examining central 5-HT mechanisms is that of drug discrimination. This is more fully discussed in section 2.3.

1.5 BENZODIAZEPINES AND 5-HT

The benzodiazepines (BDZs) are a class of compounds that have been shown to possess anticonvulsant, anxiolytic, sedative and skeletal muscle relaxant properties. A review of their clinical and experimental pharmacology has been given by Zbinden and Randall (1967) and more recently by Haefely (1983). For much of the time that they have been in clinical use very little was known about the mechanisms by which BDZs worked, but a number of recent discoveries have led to a much greater understanding of their actions. In 1975 Costa et al. and Haefely et al. showed that BDZs facilitated the action of the inhibitory transmitter gamma aminobutyric acid (GABA). This facilitation of GABAergic transmission is caused by an increase in the frequency of chloride channel openings in response to GABA (Study and Barker, 1982). The most recent advance in our understanding of the pharmacology of BDZs has been the demonstration of specific BDZ receptor sites. This area has recently been reviewed by Richards and Mohler (1984).

1.5.1 Benzodiazepine receptors

The possibility that BDZs facilitate GABA transmission by interacting with their own receptor was first suggested by the pronounced structure-activity relationship among this class of compounds. Mohler and Okada (1977) and Braestrup and Squires (1977) used the radioligand technique to show that [^{3}H]-diazepam bound with high affinity to specific sites in rat brain and that binding affinities and in vivo potencies of BDZs showed a good correlation. The BDZ binding sites are highly specific for BDZs and drugs with similar pharmacological properties, but not for other sedative

agents such as barbiturates or meprobamate (Richards and Mohler, 1984).

The highest densities of BDZ receptors are found in the cortex, cerebellum, amygdala, hippocampus and hypothalamus, while the lowest levels are found in the thalamus, pons and medulla (Mohler et al., 1978). Using photoaffinity labels for the BDZ receptor and an immunocytochemical stain for GAD, the BDZ receptors were found to be localised in areas of synaptic contact, many of which were GABAergic (Mohler et al., 1981).

The GABA receptor exists as at least two subtypes, designated $GABA_A$ and $GABA_B$ (for review see Bowery et al., 1984). The BDZ receptor is linked to the $GABA_A$ subtype only, and theoretical models for this association have been put forward by Polc et al. (1983) and Olsen (1981). The two receptors seem to exist as part of a supramolecular complex together with the chloride channel protein. Activation of BDZ receptors by agonists enhances the coupling between the GABA and BDZ receptors and increases the affinity of the GABA receptor for GABA. This increases the frequency of chloride channel openings by the GABA receptor. Activation of the BDZ receptor without the presence of GABA has no effect on chloride channel opening.

There are three groups of compounds which interact with the BDZ receptor. These are agonists, which include the BDZs, antagonists, such as Ro 15-1788, which have no action themselves but prevent access to the receptor site, and the inverse agonists (Richards and Mohler, 1984). This latter group have pharmacological actions which are the opposite of the BDZs, and in man induce panic attacks (Braestrup and Nielsen, 1982). Their action is antagonised by the

BDZ receptor antagonists, indicating that they act through the same receptor as the BDZs (Nutt et al., 1981) and probably decrease the coupling of the GABA and BDZ receptors and thus decrease the affinity of the GABA receptor for GABA (Polc et al., 1983).

As BDZs have been shown to exert their primary effects via an interaction with their own receptor, wnich is closely linked to the $GABA_A$ receptor, any interaction between BDZs and 5-HT is most likely to be related to a GABA-5-HT interaction. However, the possibility that there exist BDZ receptors not associated with $GABA_A$ receptors cannot be ruled out.

1.5.2 5-HT and the anxiolytic actions of benzodiazepines

Evidence of a role for 5-HT in the anxiolytic effects of BDZs was provided by Stein et al. (1975, 1977). They showed that the decrease in noradrenaline turnover induced by oxazepam showed tolerance, as did the sedative effects of the drug. In contrast, the decrease in 5-HT turnover persisted, as do the anxiolytic effects of BDZs (Goldberg et al., 1967). The evidence for a direct role of 5-HT in anxiety and in the anxiolytic effects of BDZs has recently been reviewed by Gardner (1985) and by Iversen (1984) who conclude that while there is evidence for an interaction between BDZs and 5-HT, more work is needed to confirm a role for 5-HT in anxiety. One of the difficulties in this area is the use of animal models to study a complex state such as anxiety, and the presence of functionally opposed 5-HT pathways in these models (Gardner, 1985). Little consideration of the role of different 5-HT receptors has been made, but the recent use of TVX Q 7821, a compound which is equipotent with diazepam in animal models of anxiety and which binds potently

to the 5-HT_{1A} site but not to the BDZ site (Dompert et al., 1985), may resolve some of the inconsistencies of earlier studies.

1.5.3 Effect of benzodiazepines on in vitro models of 5-HT activity

Most of the work studying the interaction between BDZs and 5-HT has been of a biochemical nature and, as indicated above, generally shows that BDZs decrease 5-HT activity in the CNS. Gallager (1978) applied BDZs iontophoretically and found that in conjunction with GABA they decreased the firing rate of dorsal raphe cells, but that they had no effect when given alone. Saner and Pletscher (1979) provided evidence that BDZs reduced 5-HT turnover (as measured by 5-HIAA:5-HT ratio) via a GABAergic mechanism, a conclusion supported by the work of Collinge et al. (1983) who showed that the effect of diazepam was reversed by picrotoxin and increased by ethanolamine-O-sulphate. Jenner et al. (1981) found that a number of BDZs decreased 5-HT turnover without altering synthesis, although using progabide, a GABA agonist, Scatton et al. (1982) found that both synthesis and utilisation were reduced.

Thus, there is general agreement that BDZs reduce 5-HT activity via a GABAergic mechanism, in agreement with the conclusion of Ungerstall et al. (1981) that all BDZ receptors are linked to $GABA_A$ receptors. However, some recent studies investigating an interaction between BDZs and a 5-HT receptor mediated response, namely the head-twitch, have been far less consistent, and may even challenge the conclusion of Ungerstall et al. (1981).

1.5.4 Effect of benzodiazepines on the head-twitch response

The work of Nakamura and Fukushima (1976, 1977, 1978a, 1978b)

indicates that BDZs can induce head-twitches when given alone, and will potentiate the head-twitches induced by mescaline, or by 5-HT given intracerebrally. These effects are not reversed by GABA antagonists, but are antagonised by 5-HT antagonists, and the potentiation of head-twitches is reversed by pretreatment with 5,6-dihydroxytryptamine. These results would suggest that BDZs are potentiating 5-HT agonist-induced head-twitches by potentiating postsynaptic 5-HT mechanisms. It would seem that this effect is independent of GABA receptors as it is not affected by the $GABA_A$ antagonist bicuculline. However, Handley and Singh (1984b, 1985) find that bicuculline antagonised the 5-HTP-induced head-twitch while $GABA_A$ agonists potentiated this response. In contrast, GABA itself, and aminooxyacetic acid, which increases GABA concentrations, inhibited the head-twitch response to 5-HTP. Metz et al. (1985) found that the $GABA_B$ agonist baclofen decreased the response to 5-HTP, but did not affect the response to 5-MeODMT. After chronic dosing, responses to both agents were potentiated. This was interpreted as an inhibition by baclofen of 5-HT release, as 5-HTP requires decarboxylation to 5-HT which is then released from presynaptic sites. Schlicker et al. (1984) have demonstrated that GABA can inhibit 5-HT release from cortical brain slices by a presynaptic $GABA_B$ mechanism.

There is thus a slight discrepancy in the results, as the biochemical data indicates that $GABA_A$ mechanisms can decrease 5-HT activity, but the results of both Handley and Singh (1984) and Nakamura and Fukushima (1977) indicate that both a $GABA_A$ and a BDZ mechanism will potentiate a 5-HT receptor mediated response. As Handley and Singh (1984) used 5-HTP this may mean that presynaptic

activity is not important in the action of this agent.

There would thus appear to be two components to the actions of BDZs on the 5-HT system in the CNS. The first is mediated via a GABAergic mechanism and results in decreased 5-HT neuronal activity. The second aspect seems to be a potentiation of postsynaptic mechanisms that is independent of GABA, although GABAergic mechanisms may also play a role in this.

1.6 ACETYLCHOLINE AND 5-HT

Unlike the interaction between the BDZs and 5-HT, very little published data is available on interactions between the cholinergic and 5-HT systems in the CNS. That such an interaction occurs seems likely as there is a widespread occurrence of 5-HT terminals in areas receiving a cholinergic input, such as the cerebral cortex and striatum (Anden et al, 1966). Also, as already indicated in section 1.1, both acetylcholine (ACh) and 5-HT seem to be important transmitters in the SCN. Some reports do exist, however, which indicate that an interaction between 5-HT and ACh is functionally important.

Work by Ennis and Cox (1982) provided evidence that a cholinergic interneurone mediated the effect of tryptamine on 5-HT release from hypothalamic slices, as the tryptamine effect was antagonised by atropine. Hery et al. (1977a) had already demonstrated the presence of inhibitory muscarinic receptors in the hypothalamus but whether or not this interaction is part of the control mechanism for circadian rhythms in the SCN is not known.

An involvement of striatal 5-HT in motor effects of organophosphate anticholinesterases has been demonstrated by Fernando et al. (1984). Acetylcholine is an important transmitter in the striatum (Bartholini, 1980), and administration of various anticholinesterases to rats at sub-convulsive doses induced motor effects such as tremor and hind-limb abduction (Fernando et al., 1984). At the same time 5-HT turnover in the striatum was increased and both behavioural and biochemical changes were antagonised by atropine. Cholinomimetics have been shown to induce these same behavioural effects (Cox and Potkonjak, 1970; Fernando et al.,

1984). Thus ACh may here play a facilitatory role to 5-HT. However, the opposite effect has been demonstrated by Ladinsky et al. (1981), who found elevated ACh levels in the striatum following treatment with 5-HT agonists. Potentiation of 5-HT transmission by a 5-HT uptake inhibitor has also been shown to potentiate salivation and tremor induced by oxotremorine, arecoline, or eserine (Ogren et al., 1985). There would thus appear to be a close relationship between these two transmitters in this brain region.

2 ESTABLISHMENT OF MEASURES OF 5-HT RECEPTOR ACTIVITY

2.1. MATERIALS

2.1.1 Animals

In all experiments reported in this chapter, male CFLP mice (25-40g) or male Wistar rats (250-300g at the start of the experiment) were used. They were housed in the stock rooms of the University of Bath animal house on a 14h light-10h dark cycle with lights on at 0500 hours. Food and water was freely available at all times.

2.1.2 Drugs

Dose volumes of $10ml.kg^{-1}$ and $2ml.kg^{-1}$ were used for mice and rats respectively and all drugs were given by the intraperitoneal route unless otherwise stated. Drugs were dissolved or suspended (after ultrasonification) in 0.9% saline, except pirenperone and pindolol. Pirenperone was dissolved in citrate-phosphate buffer (pH 6.4) while pindolol was dissolved in the minimum volume of methanol and made up to volume with saline. The doses given in the text refer to the salt if appropriate.

The drugs used in these experiments, with abbreviation (if used), and source given in brackets, were : 5-hydroxy-L-tryptophan (5-HTP; Sigma), 5-methoxy-N,N-dimethyltryptamine (5-MeODMT; Sigma), quipazine maleate, mescaline HCl (Sigma), 8-hydroxy-2-(di-n-propylamino)tetralin HBr (8-OHDPAT; Research Biochemicals Inc.), 5-methoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole (RU 24969; Roussel Uclaf), pirenperone (Janssen Pharmaceuticals), (+) and (-) pindolol (Sandoz Ltd.), methergoline, meta-triflouromethylphenyl-piperazine HCl (TFMPP; Research Biochemicals Inc.), dexamphetamine sulphate (Sigma).

2.2 5-HT AGONIST INDUCED HEAD-TWITCH AND SYNDROME

2.2.1 Introduction

When given to a wide range of animal species, drugs that increase levels of 5-HT in the CNS or act as direct 5-HT receptor agonists induce a range of characteristic behaviours (Jacobs, 1976). These consist of a rapid side to side motion of the head which closely resembles the pinna reflex, called a head-twitch, and a series of symptoms which collectively are refered to as the 5-HT syndrome. These symptoms are a resting tremor of the head and shoulders, reciprocal movements of the fore-limbs which has been described as "piano-playing" but is generally referred to as fore-paw treading, a slow side to side movement of the head called head-weaving and a splaying of the hind-limbs or hind-limb abduction.

The evidence for the mediation of these behavioural effects by one or other of the 5-HT receptors so far described has already been summarised in section 1.4, but even a brief study of this field will show that the 5-HT-agonist induced head-twitch is the best categorised of all the models purporting to reflect activity at a 5-HT receptor; in this case the 5-HT_2 subtype. The situation with the 5-HT syndrome is far less clear, but as it is generally induced by the same agents as the head-twitch they have been studied together in the following experiments. Of the drugs used, 5-MeODMT and 5-HTP have been studied most extensively, while mescaline and quipazine were also studied to provide a greater variety of agonists. This was felt necessary as none of the agents used are selective post-synaptic 5-HT agonists.

The direct 5-HT agonist 5-MeODMT does not appear to discriminate between 5-HT_1 and 5-HT_2 receptors. It has been shown to induce head-twitches in mice (Friedman and Dallob, 1979) and using the drug discrimination paradigm it is found to generalise to both LSD and quipazine (Rosencrans and Glennon, 1979; Glennon et al., 1982), drugs whose stimulus properties depend primarily on the 5-HT_2 receptor subtype (Colpaert and Janssen, 1982; Friedman et al., 1984). On the other hand, 5-MeODMT is a potent displacer of ligands for the 5-HT_1 recognition site (Martin and Sanders-Bush, 1982), induces contralateral rotation in unilaterally 5,7-dihydroxytryptamine-lesioned rats, a model thought to reflect activity at the 5-HT_1 receptor (Blackburn et al., 1984), and also generalises to the 5-HTP discriminative stimulus, which appears to be mediated via the 5-HT_1 receptor (this thesis). The 5-HT_1 site has also been identified with pre-synaptic receptors, and 5-MeODMT potently depresses the firing rates of serotonergic neurones (de Montigney and Aghajanian, 1977) and has also been shown to inhibit the release of 5-HT from synaptosomes (Martin and Sanders-Bush, 1982).

5-HTP is the natural precursor of 5-HT and is therefore an indirect agonist as it requires decarboxylation to 5-HT before it can act at any receptor sites. 5-HT itself will produce head-twitches, but it needs to be given directly into the CNS (Suchowsky et al., 1969) as unlike 5-HTP it does not pass the blood-brain barrier (Udenfriend et al., 1957). There is considerable evidence to suggest that the head-twitches induced by 5-HTP in both rats and mice result from a central action of 5-HT. Thus the decarboxylase inhibitor, methyldopa, antagonises the action of 5-HTP (Corne

et al., 1963) whereas the peripheral decarboxylase inhibitor, carbidopa, does not (Matthews and Smith, 1980).

Quipazine will also induce head-twitches in both rats and mice (Matthews and Smith, 1980; Malick et al., 1977) which strongly suggests that it is a 5-HT_2 agonist. Further evidence for this comes from its use as a discriminative stimulus where its properties are consistent with its mediation by the 5-HT_2 receptor (Friedman et al., 1984). There is also some evidence for quipazine having activity at pre-synaptic sites where, unlike 5-MeODMT, it acts as an antagonist (Martin and Sanders-Bush, 1982; Schlicker and Gothert, 1981).

Much less is known about the properties of mescaline as a 5-HT agonist. Corne and Pickering (1967) showed that it could induce head-twitches but it is not a very potent displacer of binding to the 5-HT_2 site, being some twenty times less potent than 5-HT, which is itself a poor ligand for this site (Leysen et al., 1982).

2.2.2 Methods used in the mouse

Before using any of these agonists, the time of peak activity following i.p. administration was determined, using doses obtained from previous reports and the time-response curves are shown in Fig.1. All subsequent experiments were carried out at the times of peak effect, which are detailed in the following section.

a) 5-MeODMT

Mice were given 5-MeODMT and immediately placed in one half of a plastic cage measuring 30x15x15 cm which had been divided in half with a metal partition. This enabled two mice to be studied at the same time. The mice were left in these boxes for 1 min

before being observed for a period of 3min and the number of head-twitches occurring during this time was recorded. The 5-HT syndrome was assessed in the same mice during the subsequent minute using the following rating scales:

Tremor and Fore-paw treading:	0	Absent
	1	Present
	2	Marked
Head-weaving and Hind-limb abduction:	0	Absent
	1	Equivocal
	2	Present
	3	Marked

This gives a maximum possible score for each animal of 10.

Times of administration and doses of the antagonists used are given in the results.

b) 5-HTP

Mice were administered carbidopa (25 $mg.kg^{-1}$) 20 min before receiving 5-HTP, and after a further 20 min were placed in the observation boxes described in the previous section. They were then watched for 3 min and the number of head-twitches and the severity of the 5-HT syndrome recorded as before.

c) Quipazine

The method given for 5-MeODMT was also followed for quipazine.

d) Mescaline

Mice were administered mescaline 30 min before being placed in the observation boxes and watched for 3 min. The number of head-twitches during this period was recorded.

Figure 1. Time course of activity for induction of head-twitches by 5-HT agonists in mice. All values are mean ± s.e.m.

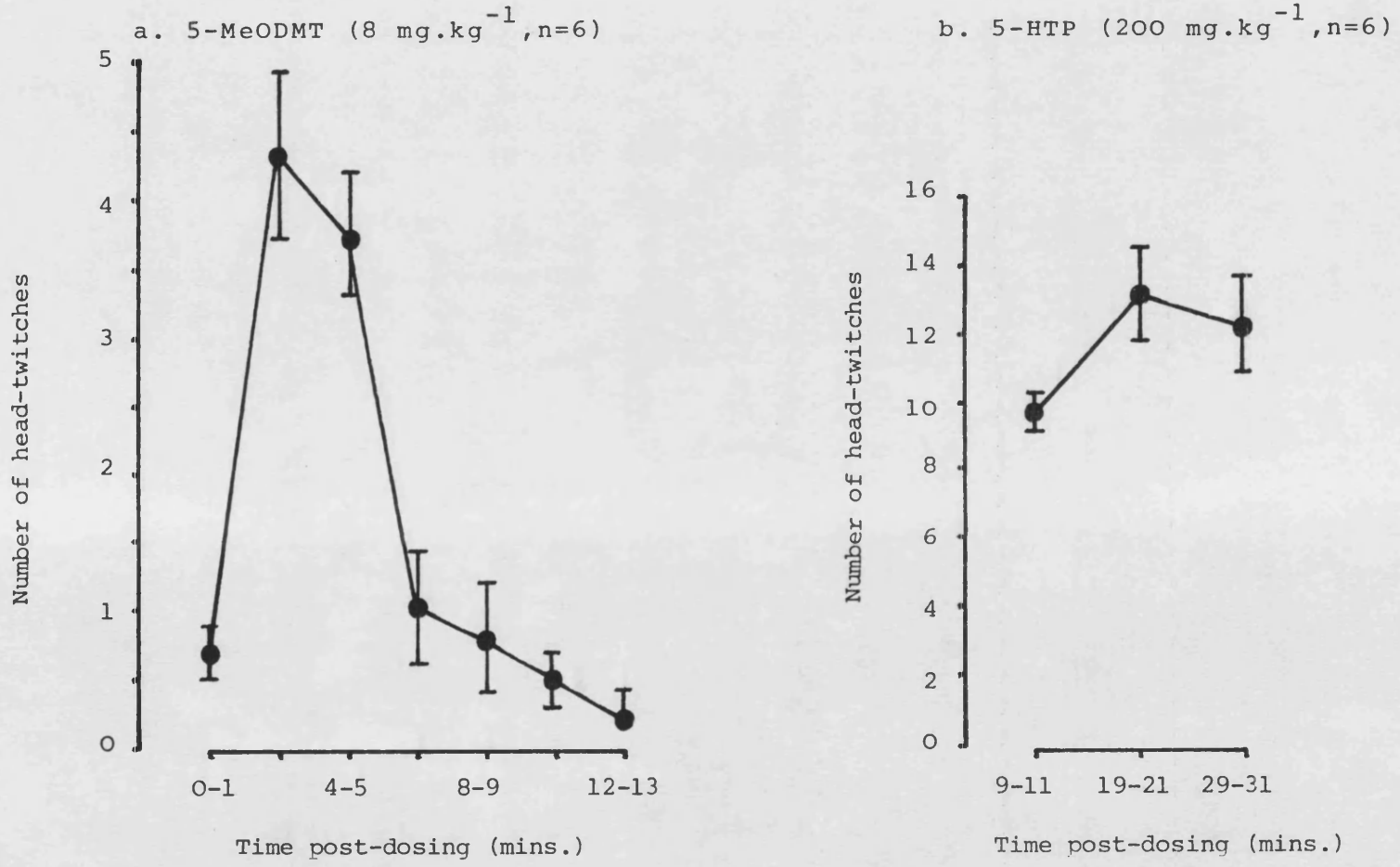
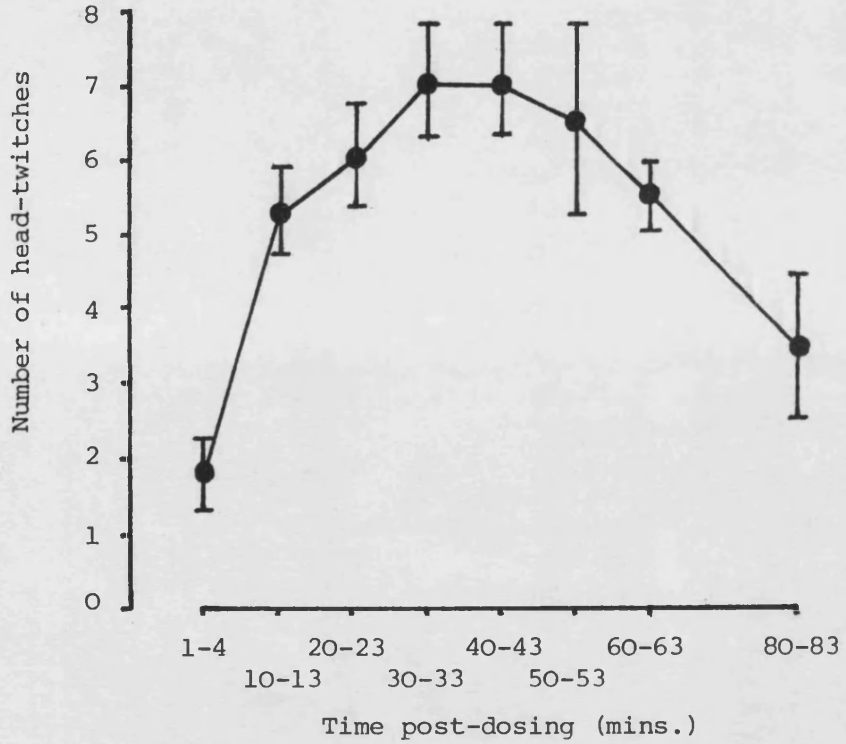
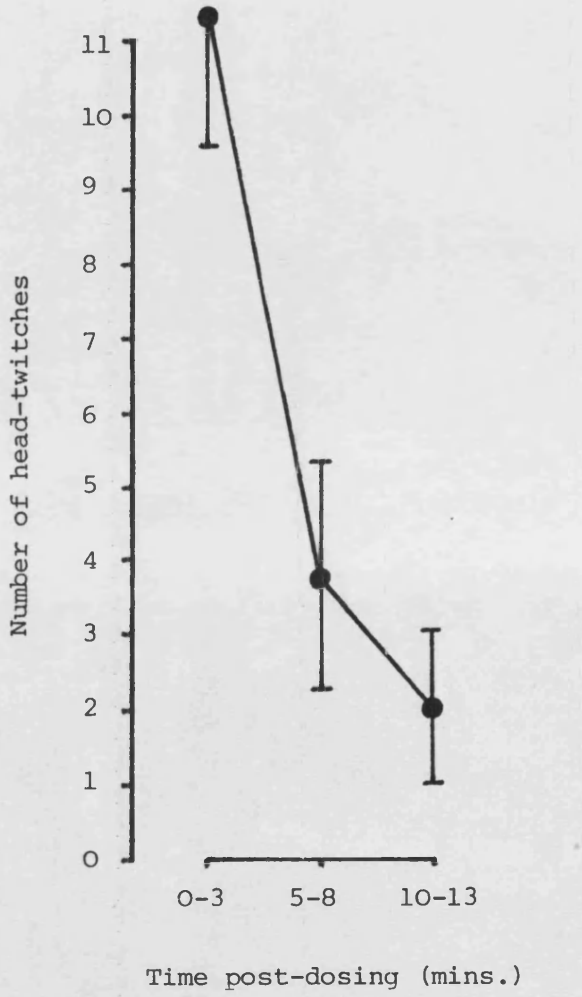


Figure 1. Continued

c. Mescaline (20 mg.kg^{-1},n=4)



d. Quipazine (20 mg.kg^{-1},n=3)



2.2.3 Dose response curves for 5-HT agonists in the mouse

Log-dose-response curves for induction of head-twitches for each of the four agonists used are shown in Fig.2. Clearly, 5-MeODMT is the most potent, followed by quipazine, mescaline and 5-HTP. Another feature of the dose-response curves is that both quipazine and mescaline have a lower maximum effect than either of the other two agents. This is possibly as a result of partial agonist activity, a property which quipazine has been shown to exhibit at peripheral 5-HT receptors (Lansdown et al.,1980).

Dose-response curves for inducing the 5-HT syndrome are only given for 5-MeODMT (Fig.3) as none of the other agonists produced high levels of this measure of 5-HT receptor activity. Using this rating scale 5-MeODMT was found to be approximately equipotent as an inducer of both the head-twitch and the 5-HT syndrome. When the highest dose of 5-HTP was used a maximum score of 5 was obtained for the syndrome. The severity of the syndrome was not recorded for either of the other two agonists.

2.2.4 Effect of antagonists in the mouse

Only a limited number of 5-HT antagonists were used against the head-twitches as there is already a large body of published work covering this field which demonstrates the mediation of this behaviour by the 5-HT_2 receptor (e.g. Yap and Taylor, 1983; Matthews and Smith, 1980), and these brief experiments were performed to confirm the previous results.

The effects of methergoline, pirenperone and pindolol on the head-twitches and syndrome induced by 5-MeODMT, and of methergoline on the head-twitches induced by 5-HTP, were examined. All of the

Figure 2. Dose-response curves for induction of head-twitches in mice.

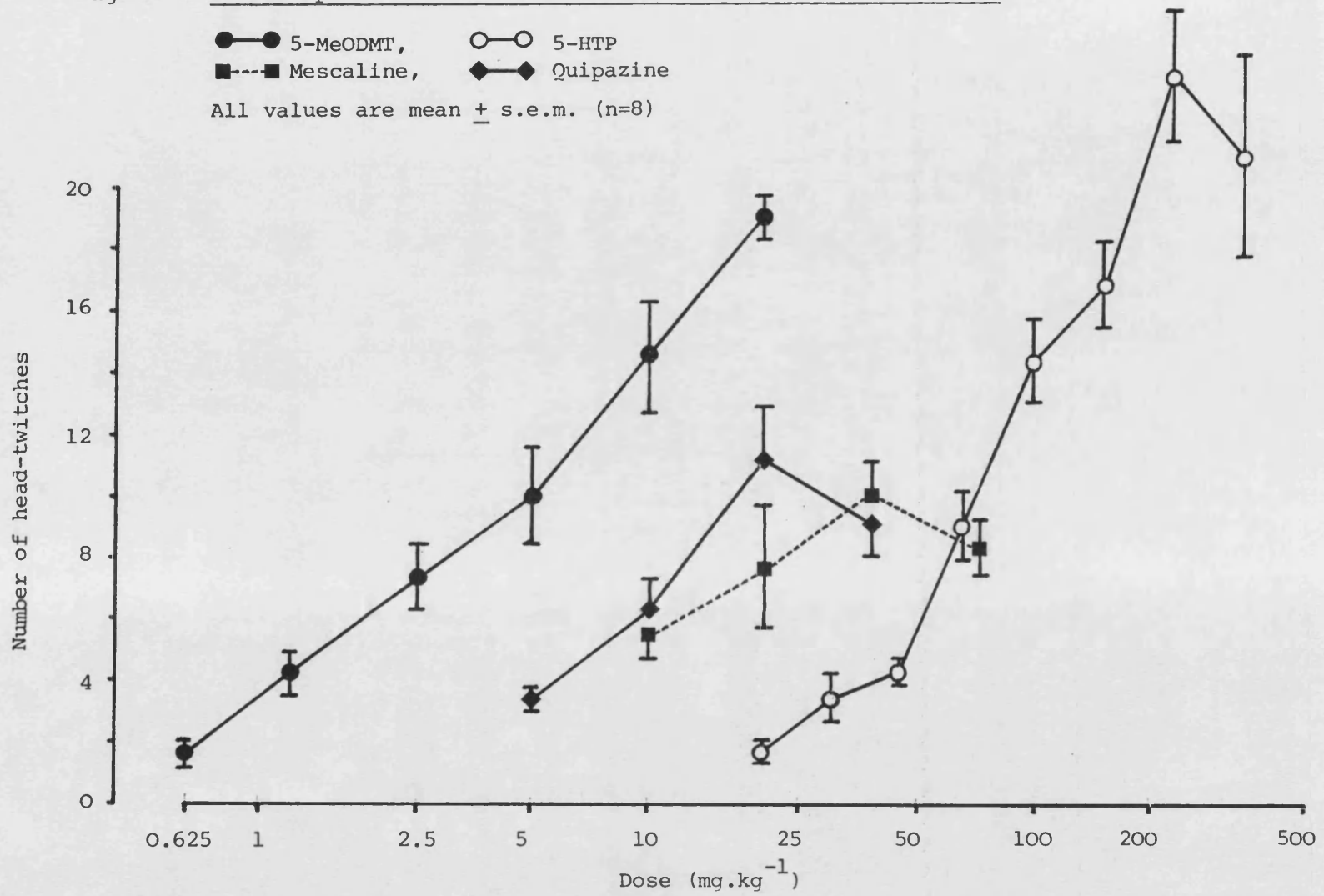
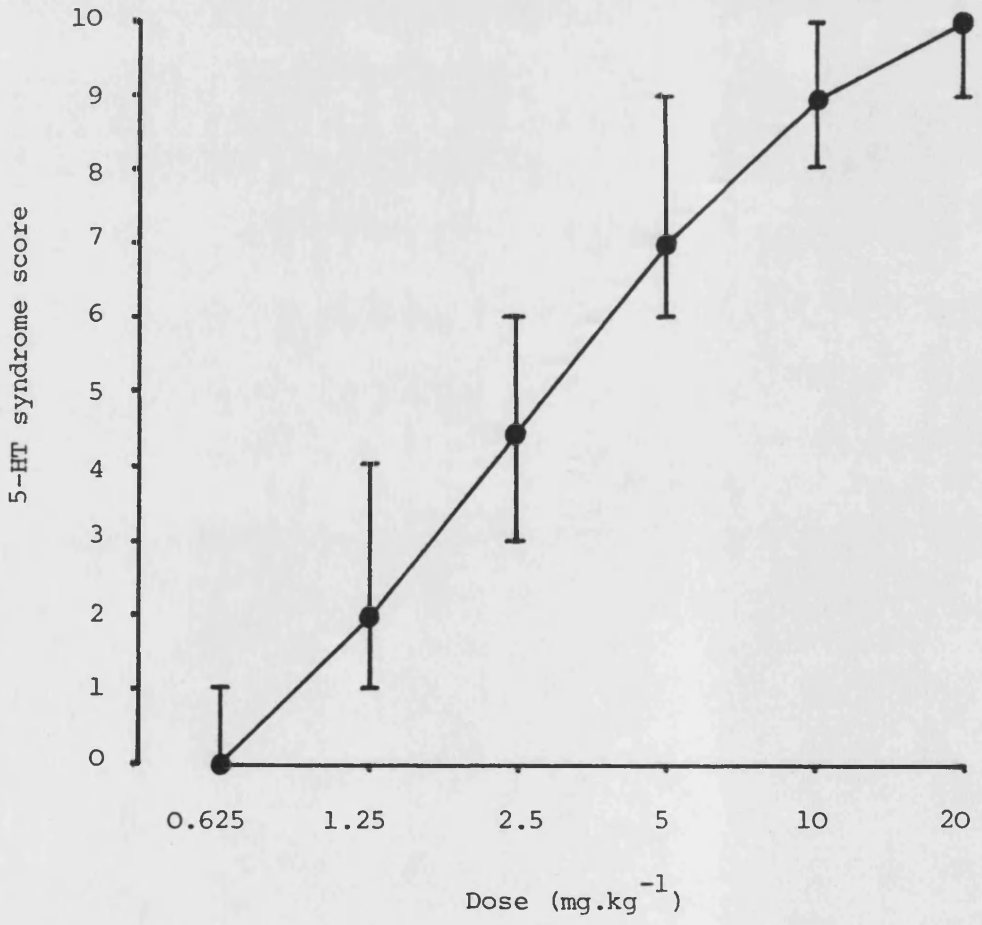


Figure 3. Dose-response curve for induction of 5-HT syndrome by 5-MeODMT in mice.

All values are median and range (n=8)



antagonists were administered 60 min before the 3 min observation period using the methods given above for these two agonists. Doses of 200 $mg.kg^{-1}$ and 16$mg.kg^{-1}$ were used for 5-HT and 5-MeODMT respectively.

The results for methergoline and pirenperone are shown in Figs. 4 and 5, and it is clear that the head-twitch and the syndrome respond differently as it is possible to obtain maximum inhibition of the former response with hardly any effect on the latter with both pirenperone and methergoline. Careful analysis of the syndrome response following pirenperone indicated that the inhibition was due to attenuation of the head-weaving and fore-paw treading only. Tremor and hind-limb abduction were not affected at even the highest dose used. Pirenperone was found to be approximately 200 times more potent than methergoline. In contrast to these results both isomers of pindolol were without effect against both the head-twitch and syndrome induced by 5-MeODMT, as shown in Table 1.

Table 1 The effects of the isomers of pindolol on behavioural responses to 5-MeODMT

Treatment	Dose ($mg.kg^{-1}$)	n	Head-twitches (mean ± s.e.m.)	Syndrome (median and range)
Saline	-	8	16.1 ± 1.4	9 (8-9)
(-)-Pindolol	4	8	15.9 ± 1.3	8 (7-10)
(+)-Pindolol	4	8	15.0 ± 2.0	9 (8-9)

Figure 4. Inhibition of 5-HTP-induced head-twitches and of 5-MeODMT-induced head-twitches and syndrome by methergoline in mice.

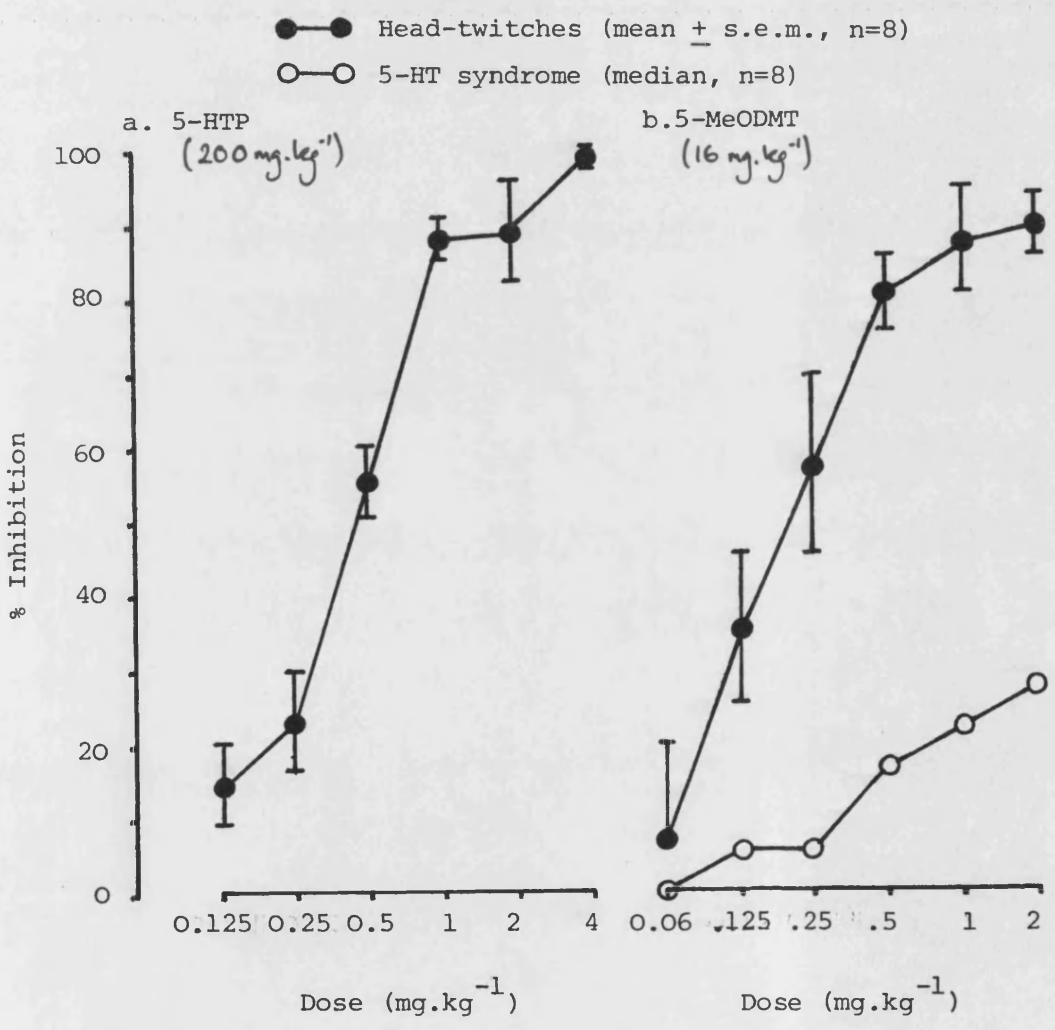
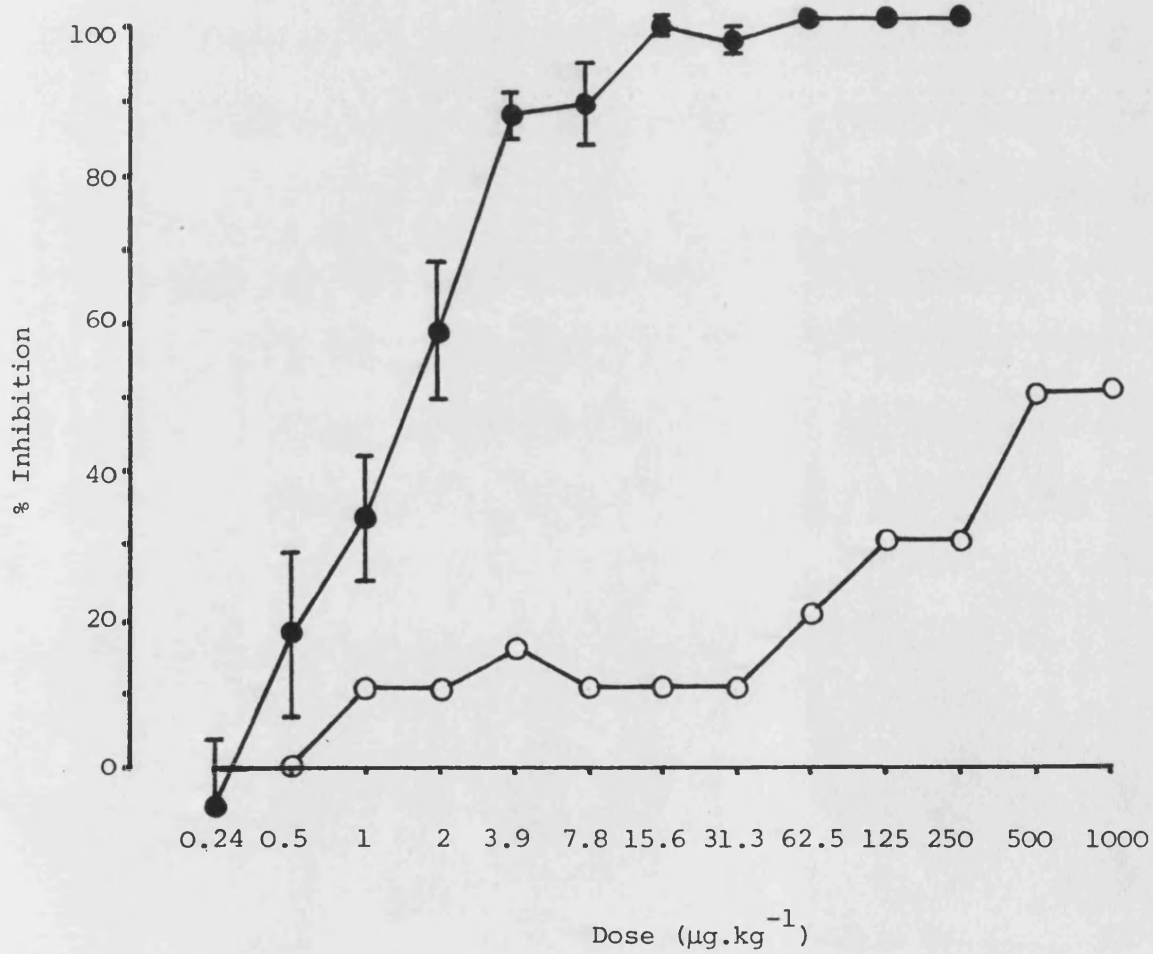


Figure 5. Inhibition of 5-MeODMT-induced head-twitches and 5-HT syndrome by pirenperone.



2.2.5 Methods used in the rat

Time-courses of action for the agents used in this section are shown in Figs. 6 and 7. The effects of 5-HTP were not studied at the time of peak effect as this work was designed to complement the drug discrimination work described in section 2.3.

a) 5-MeODMT

As 5-MeODMT was not found to induce head-twitches in the rat only the syndrome was measured.

Groups of rats received 5-MeODMT and were immediately placed individually in plastic cages (40x25x25cm). Signs of the 5-HT syndrome were scored using the same rating scale as that used for mice during the period 7-8 min. post-dosing.

The effect of pirenperone, administered 60 min. prior to the observation period, on the syndrome induced by 5 $mg.kg^{-1}$ 5-MeODMT was also studied.

b) 5-HTP

Groups of rats received carbidopa (25 $mg.kg^{-1}$) followed 30 min. later by 5-HTP. After a further 30 min. they were placed in the observation boxes and watched for 10 min. during which time the number of head-twitches was recorded. At the end of this period the 5-HT syndrome was scored.

The effect of pirenperone (60 min. before the observation period) on the behaviours induced by 100 $mg.kg^{-1}$ 5-HTP was also studied.

Figure 6. Time-course of activity for induction of head-twitches by 5-HTP in the rat. (100 mg.kg⁻¹)

All values are mean ± s.e.m., n=6.

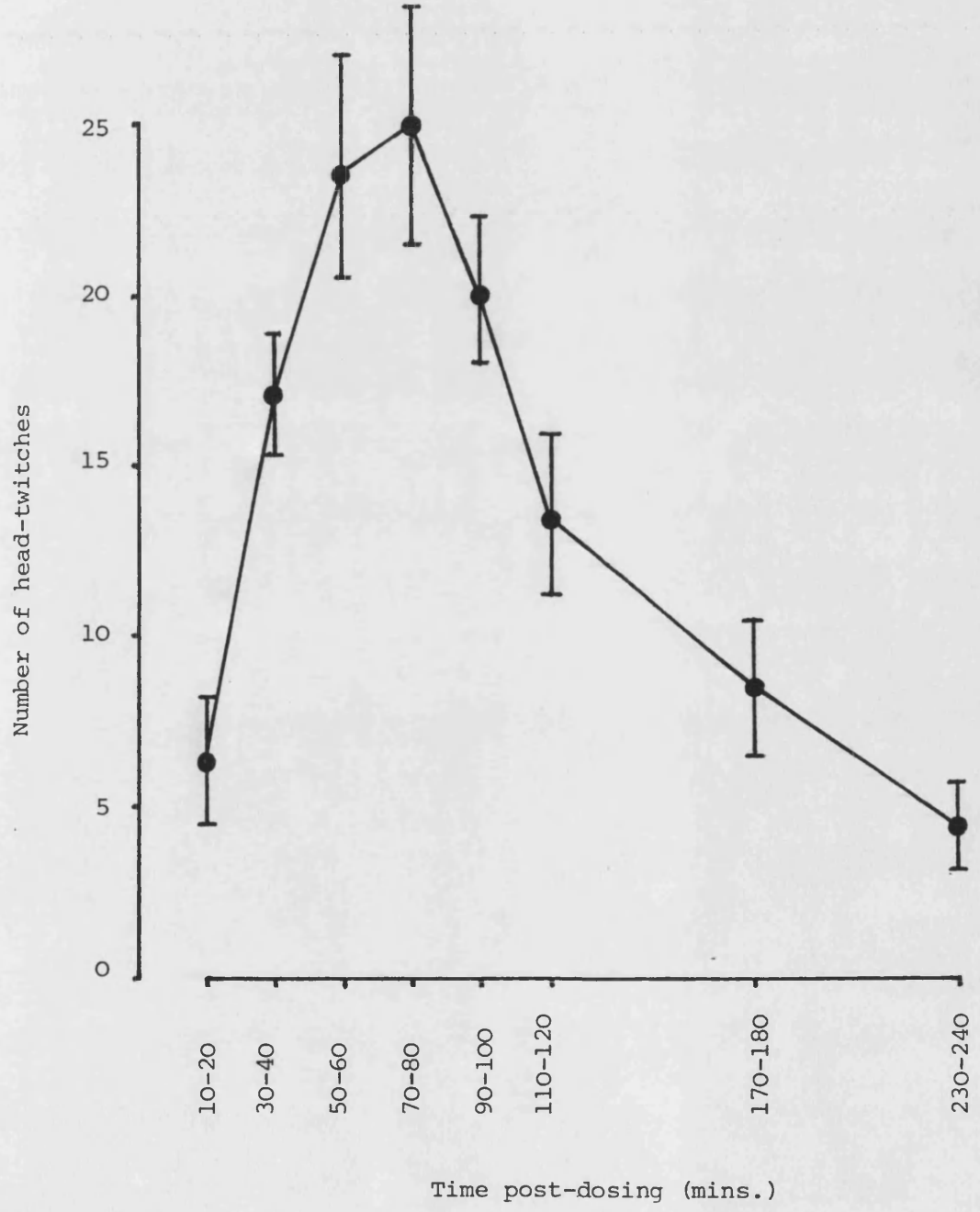
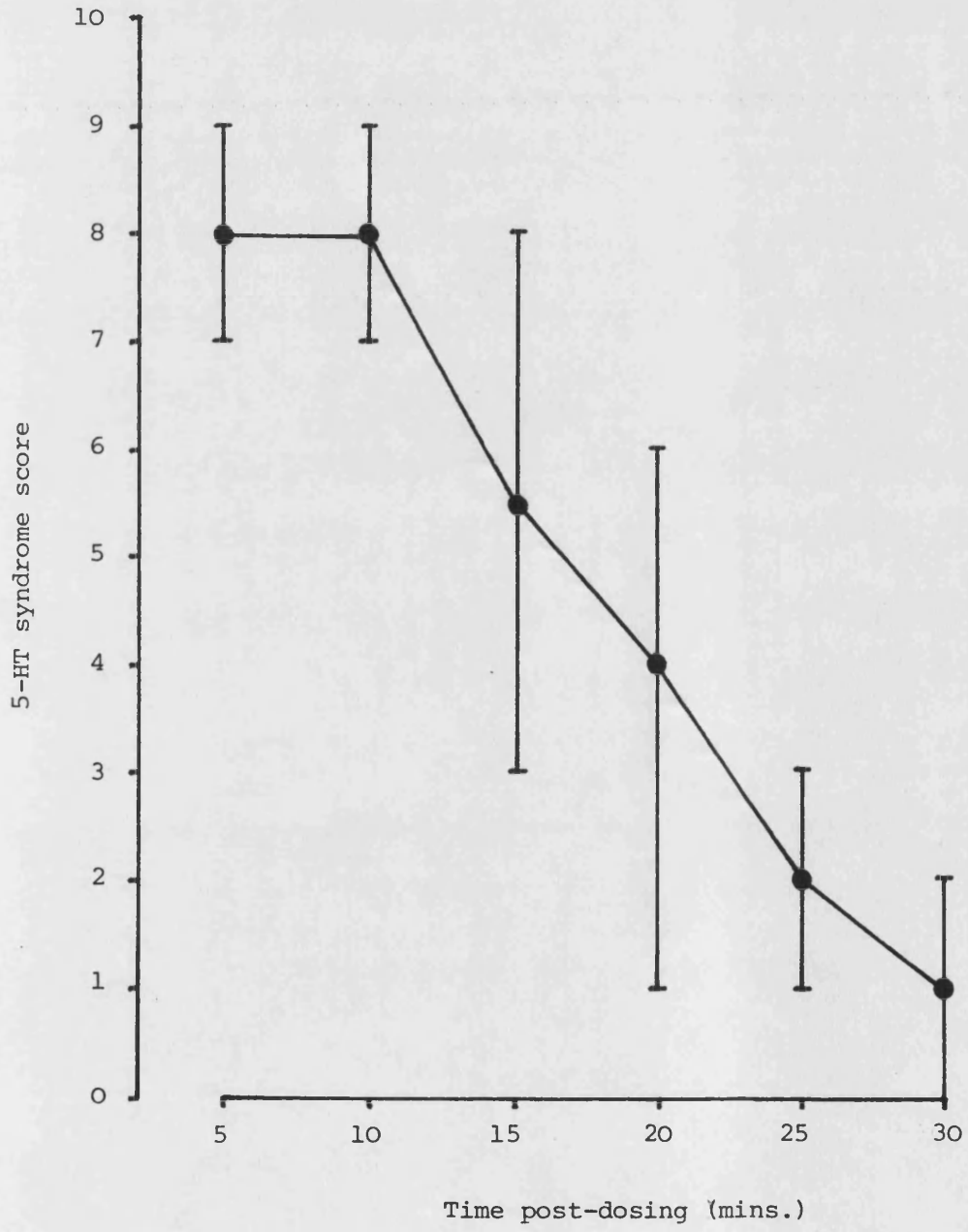


Figure 7. Time-course of activity for induction of the 5-HT syndrome by 5-MeODMT in rats. (5 mg.kg^{-1})

All values are median and range, n=8.



2.2.6 Dose response curves for 5-HT agonists in the rat

The head-twitches induced by 5-HTP were found to be dose dependent (Fig. 8a) but at none of these doses were there any marked signs of the 5-HT syndrome.

In contrast, following administration of 5-MeODMT, signs of the 5-HT syndrome rapidly appeared, reaching a peak between 7 and 8 min. post-dosing. This effect was dose dependent and had an ED_{50} of approximately 2 $mg.kg^{-1}$ (Fig. 8b).

2.2.7 Effect of antagonists in the rat

Pirenperone was found to be a potent antagonist of the 5-HTP induced head-twitch with an ED_{50} of 2.3 $\mu g.kg^{-1}$ (Fig. 9). A dose of 200 $\mu g.kg^{-1}$, however, was only able to inhibit the syndrome induced by 5-MeODMT by 50%.

Figure 8. Dose-response curves for induction of the 5-HT syndrome and head-twitches by 5-HTP and 5-MeODMT.

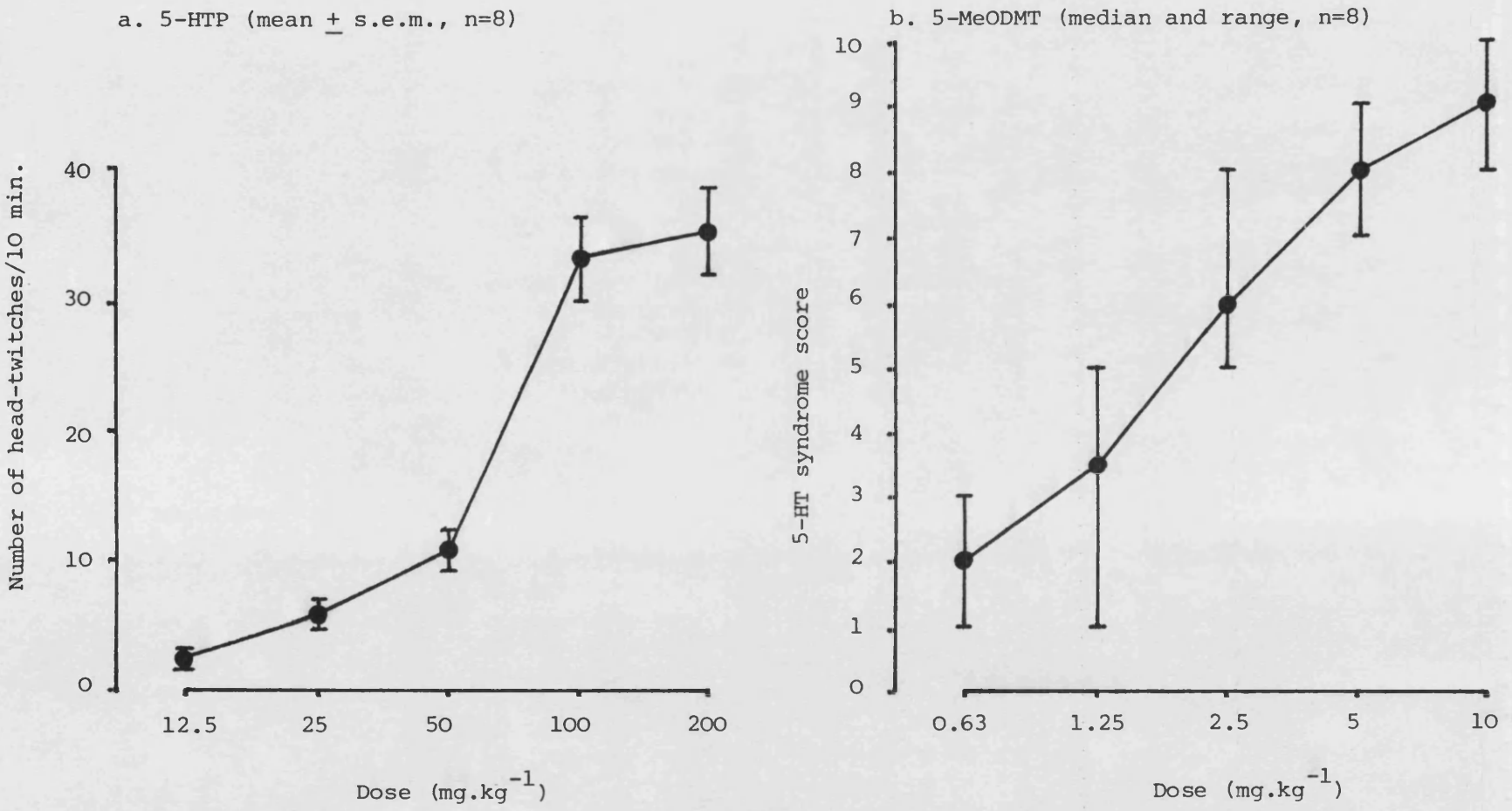
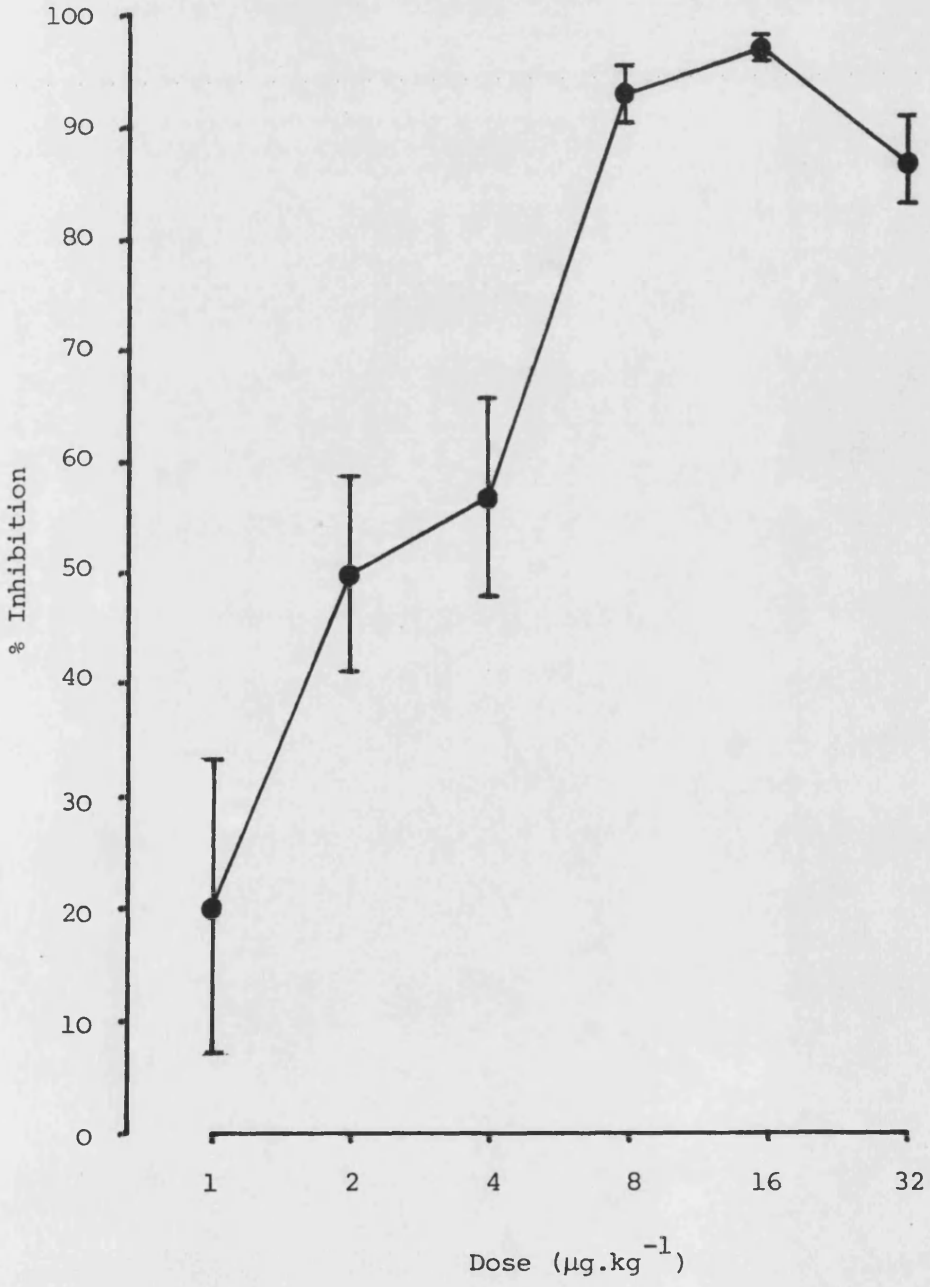


Figure 9. Inhibition of 5-HTP-induced head-twitches by pirenperone in the rat.

All values are mean ± s.e.m., n=8.

Dose of 5-HTP : 200 mg.kg^{-1}



2.3 DISCRIMINATIVE STIMULUS PROPERTIES OF 5-HTP

2.3.1 Introduction

In the following section on drug discrimination the discussion has been limited to discrimination learning, and references to state dependent learning have been avoided. Some of the terms used apply to both methodologies, but these have been used only in the context of drug discrimination. An example of this is the term drug state which is only used to imply the experimental circumstance when a subject has received drug (as opposed to saline) for training purposes. Drug discrimination and state dependent learning have some similarities, but they represent distinct properties of drugs (Colpaert, 1977). In drug discrimination learning it is assumed that animals can perceive the different effects of drug or saline injections and learn to make choices on the basis of that difference. State dependent learning suggests that responses learnt in one state, i.e. drug or saline, cannot be performed in the other as their recall is only possible under the same conditions, and that animals need not be able to consciously perceive the effects of the drug. Higher doses are generally required for state-dependent effects of drugs.

Drug discrimination is thus a method which allows the study of interioceptive effects of drugs in animals, usually at doses that do not produce any visible external signs that the animal has received an active drug. Typically, in these experiments, one is asking the animal to discriminate between two states, one following saline injection and one following drug injection. To show this discrimination, subjects are trained to make one response in the

saline condition, and another in the drug condition by the reinforcement of correct responses. These two responses and the reinforcement can take many forms but usually the responses are similar to avoid asymmetry in the experimental design, and they must be mutually exclusive. The most common types of reinforcement used are food reward, which necessitates keeping the animal below its normal free-feeding weight, and shock escape or avoidance. Thus, subjects are trained to press one of two adjacent levers to obtain food reward in the saline state, and the other to obtain reward in the drug state, or to turn in one direction to escape shock in a T-maze under one condition and the opposite direction in the other.

This training continues until the animals reach a certain standard of performance, usually by fulfilling a criterion which is set before the start of the experiment. The use of a criterion of discrimination is used as animals rarely, if ever, show 100% performance in the task and so a predetermined level is necessary at which the animal is said to have learnt to discriminate between the two training states. This must be high enough to avoid attainment by chance, but low enough to be reached by most animals after sufficient training. A level of 80% is usually adopted. When the T-maze is used, as in the experiments reported in this thesis, an 80% criterion means that an animal has made the correct choice on the first trial during 8 out of 10 consecutive sessions. Training sessions are carried out daily and consist of a number of trials in the maze where the animal makes a direction choice. Only the choice made on the first trial in each session can be used as a measure of performance, as choices on subsequent trials will obviously be influenced by the outcome of the animals choice

during that first trial (Zenick and Green,1978).

The speed at which animals learn the discrimination depends on a number of factors which include the discriminability of the drug, the dosage used and the training technique employed. A large number of drugs have been shown to possess the ability to provide a cue and the stimuli provided by these agents seem to have a number of things in common. An action on the CNS appears to be an important attribute (Barry, 1974) but is not absolutely essential, although drugs with only peripheral actions are harder to discriminate than centrally acting drugs (Lal, 1977). Also the stimulus should be weak rather than punishing or reinforcing (Barry and Krimmer, 1978). It is also essential that the drug does not disrupt the ability of the animal to make a response. This is the case with 5-HTP if it is given without prior inhibition of peripheral decarboxylase (Carter et al., 1978) as the peripheral effects of 5-HT inhibit the animals ability to make the movements necessary for the response.

The training dose of the drug is probably the major variable in studies of this type and can affect discrimination in several ways. The dose used will clearly affect the strength of the effect perceived by the animal and it will also affect the sensitivity of the discrimination. Thus animals trained on a high dose will not respond to such low doses as animals trained on lower doses of the same drug. Another effect of training dose can be on the pharmacological basis of the cue properties of a drug. No drug is completely selective in its actions and lower doses will therefore be more specific to a particular drug action than higher doses (Colpaert, 1982).

The most obvious effect of different training doses, however,

is on the rate at which animals learn the discrimination task. Overton (1974) showed that there was a direct relationship between increasing drug dosage and increasing stimulus strength as determined by the number of sessions needed to train rats to criterion. As the time taken to reach criterion can be speeded up by using a higher training dose, some experimenters have used this as a method of decreasing training time for low doses. Animals are first trained on a high, easily discriminable dose, which is then lowered when animals reach criterion. Training then continues until animals again reach criterion when the process can be repeated. Using this technique it is possible to achieve not only faster training but also to obtain discrimination with doses of a drug that do not appear to possess cue properties under normal circumstances. This method has been applied with some success to LSD (Greenberg et al., 1975). A possible problem with this method is that animals will learn to discriminate a different pharmacological property of the drug than that intended, but this is offset by the large increase in sensitivity which can be obtained.

The training technique used can also influence the time taken to reach criterion, such as varying the number of trials used in each session. Barry and Krimmer (1978) report that the use of only two or three trials per session retards learning compared to the more usual practice of eight to ten. This is probably the result of incorrect choices becoming a greater percentage of the total and thus exercising greater influence in recall during subsequent sessions. The use of food-reward rather than shock-escape as the reinforcement has also been shown to affect the sensitivity of the discrimination and would thus be expected to affect acquisition of

discrimination (Barry and Krimmer, 1978).

Once discrimination has been established, experiments can be performed to find out which drugs antagonise the training drug stimulus or which drugs will substitute for the cue properties of the training drug. This latter effect is referred to as transfer or generalisation. Experimental sessions are interspersed between training sessions so that the control performance of the animals can be continuously monitored and to give the animal a series of reference states against which the test conditions can be evaluated. During each experimental session the animals are being asked to compare the test state with the two training states and to indicate, by its direction choice in the T-maze, which of the two states most resembles the test condition. It is important to realise that the animal has no way to indicate that the test condition does not resemble either of the two training states, and by the nature of the test is often impelled to make a response. This may lead to over-inclusiveness of transfer tests, as suggested by Overton (1974), but there is some evidence that if the test state resembles neither training state then the animal will choose the non-drug direction in the T-maze, even though the test drug may have discriminative stimulus properties of its own (Overton, 1966; Stewart, 1962). It has also been suggested that totally random responding during test sessions could also indicate that the test state resembles neither training state (Overton, 1966).

Using transfer and antagonism tests it has been shown that the cues produced by drugs can be highly specific to a particular pharmacological action. Overton (1982b) has identified at least twenty types of discriminably-different drugs and numerous papers

attest to the distinctiveness of the cue properties of many drugs. For example, although various types of depressant drugs will substitute for each other when trained against saline (Overton, 1966) more specific training allows rats to discriminate phenobarbital from other depressants such as ethanol, chlordiazepoxide and ketamine (Overton, 1982).

Drug discrimination can thus be a powerful tool in the study of drug actions and can be used as a very sensitive behavioural measure of drug-receptor interactions.

2.3.2 Serotonergic drugs as discriminative stimuli

The history of the discriminative stimulus properties of drugs which act on the serotonergic system of the CNS began early in the developement of the technique, due to the interest shown by workers studying the properties of hallucinogenic drugs. By its very nature it is not possible to directly measure hallucinogenic potential in animals, but a method which allows the detection of interioceptive properties of drugs would obviously prove useful.

Several hallucinogenic drugs were shown to possess cue properties around the same time. These were mescaline (Overton, 1971), psilocybin (Harris and Balster, 1971) and LSD (Hirschorn and Winter,1971) and in 1972, Schecter and Rosencrans found that rats trained to discriminate LSD from saline would choose the LSD appropriate response when given mescaline or psilocybin, but not when given amphetamine. LSD has since become something of a standard cue in discrimination studies (Stolerman and Shine, 1985). Its use in analysing mechanisms of hallucinogenic drug action has been reviewed by Appel et al., (1982) who conclude that, while it

has proved useful, transfer to the LSD cue does not predict hallucinogenic potential but rather a common action of these drugs upon a central serotonergic mechanism. Cameron and Appel (1973) and Kuhn et al., (1978) had both provided evidence that the LSD cue was mediated by a 5-HT receptor as it was potentiated by pCPA and antagonised by 5-HT antagonists but not by dopamine, α- and β-adrenergic, cholinergic or histaminergic antagonists. Glennon et al., (1980) later showed a correlation between 5-HT receptor affinity (as measured on rat fundic strip) and transfer to the 5-MeODMT cue using a series of tryptamine analogues. The conclusion of Appel et al. (1982) was largely based on the fact that drugs like quipazine would transfer to the LSD cue despite not appearing to induce hallucinations in man, and that all the drugs used had an agonist action at 5-HT receptors. This conclusion may still be in error however, as quipazine has been reported to induce mescaline-like effects in man (personal communication cited by Winter, 1979).

More recently, drug discrimination has been used to study the pharmacology of central 5-HT receptors, mainly as a result of the confusion surrounding the classification of behaviours that result from stimulation of these receptors (see Introduction). There is now substantial evidence that the LSD cue is mediated by the 5-HT_2 receptor subtype as it is antagonised by the selective 5-HT_2 antagonists pirenperone (Colpaert et al., 1982) and ritanserin (Colpaert et al., 1985). It is therefore likely that all hallucinogenic agents that transfer to the LSD cue also act via the 5-HT_2 receptor. The cue properties of quipazine have also been shown to be mediated by the 5-HT_2 receptor (Friedman et al., 1984).

The first indication of a 5-HT receptor-mediated cue that is

not based on activation of 5-HT_2 receptors came with the results of Barrett et al., (1982) and Friedman et al., (1983) who described the stimulus properties of 5-HTP. They showed that it generalised to the 5-HT releaser fenfluramine, but not to the catecholamine releaser amphetamine. They also demonstrated that the 5-HT uptake inhibitor fluoxetine would potentiate the cue but that the classical 5-HT antagonists methysergide, cyproheptidine, methergoline and methiothepin had no effect. These antagonists do block the LSD cue (Kuhn et al., 1978) which suggests that the 5-HTP cue is not mediated by the 5-HT_2 receptor. Evidence that a central receptor is involved comes from other results of Friedman et al., (1983) which show a correlation between cue potency of 5-HT and central 5-HT levels and the need for the presence of a peripheral decarboxylase innibitor.

The experiments described below were designed to show whether or not the 5-HTP discriminative stimulus was mediated by the 5-HT_1 receptor. To this end the putative 5-HT agonists 8-OHDPAT, RU 24969 and TFMPP were tested for transfer to the 5-HTP cue and the more specific 5-HT_2 antagonists used to confirm that this receptor is not involved, as recently suggested by Cunningham et al., (1985).

In their original papers Barrett et al., (1982) and Friedman et al., (1983) used operant techniques to study the cue properties of 5-HTP but this would not lend itself to the study of circadian rhythms, the primary aim of the present study. In the two-lever operant paradigm the animal works for a food reward which makes it necessary to limit the feeding of the subjects so that their bodyweight remains below normal. This is normally done by restricting feeding to a limited period of 2 hours per day, which

would be impractical with a number of groups of animals on different light-dark schedules. Another disadvantage of food reward is that drugs may affect an animals appetite and therefore the value of food as a reinforcer (Jarbe and Henriksson, 1973).

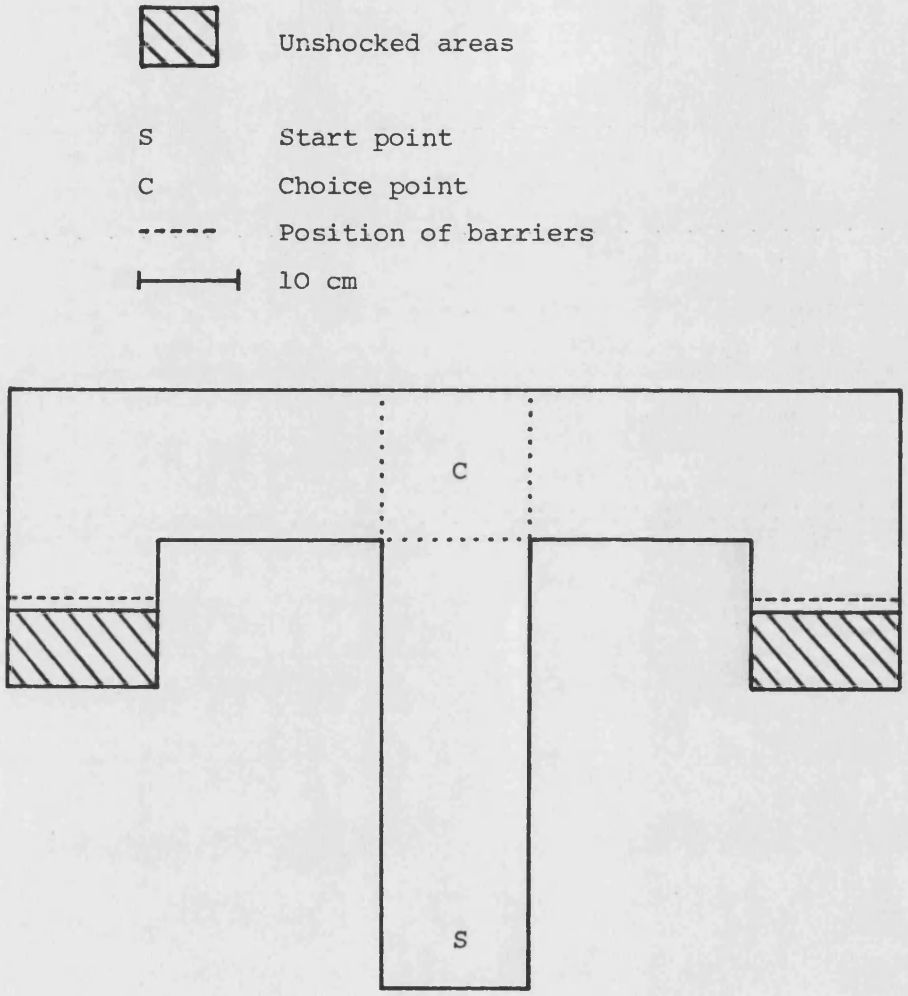
The technique chosen for this study was therefore shock escape in the T-maze. This has been used by a number of workers (e.g. Bindra and Reichart, 1966; Overton, 1966) and can produce good results although it seems to be falling out of favour (Stolerman and Shine, 1985). It can be more time consuming than the operant technique because larger numbers of animals are required as it is more prone to problems of inter-animal variation (Koek and Slangen, 1984), but it avoids problems of response probing which can occur using lever press methods (Colpaert, 1977).

A possible criticism of the previous work on the 5-HTP cue is that only those animals receiving 5-HTP also received a peripheral decarboxylase inhibitor and not those that were dosed with saline. This raises the possibility that animals are learning to discriminate the decarboxylase inhibitor from saline or that the presence of multiple cues may be affecting the results (Koek and Slangen, 1984). These problems were avoided in the following experiments by pre-treating all animals with carbidopa to provide a constant background for the 5-HTP/saline discrimination.

2.3.3 Methods

Groups of rats were trained to discriminate 5-HTP from saline using shock escape in a T-maze constructed from 7-ply plywood according to the plan shown in Fig. 10. The side walls were 30cm high and the base of the maze consisted of 3mm diameter metal

Figure 10. Plan of T-maze.



rods 1cm apart which were supplied with current from a constant current shock generator (Campden Instruments, Model 521C) fed through a shock scrambler (Campden Instruments, Model 521S). The areas shown at the end of each side-arm in Fig. 10 had a plywood floor where animals could escape the shock and were designated safe areas. It was possible to block off each of these safe areas with a wooden door so that the animal could not escape the shock in one or other of the side-arms. The safe areas were not visible to the animal from the choice point at the end of the main runway so that it had to enter the side-arm before it discovered whether or not that safe area was available.

Rats were initially trained to escape the shock by running to the open safe area of the maze. During this period a current of 0.5mA was used. Once shock escape had been established, discrimination training started and the current was lowered to 0.25mA. Training sessions were carried out on weekdays only and each session consisted of 8 trials one minute apart. On each trial, with the current already turned on, rats were placed in the main arm of the maze facing away from the choice point and allowed to run in the maze until they reached the open safe area. During each session the only data recorded was the rat's direction choice on the first trial. This was defined as the first side-arm that the rat entered completely (i.e. to the base of its tail) from the choice point.

On each training day all rats received carbidopa (25 $mg.kg^{-1}$) 60 min. before the first trial, followed 30 min. later by either 5-HTP or saline. This dosing schedule is the same as that used by Barrett et al. (1982). Analysis of the time course for head-twitches induced by 5-HTP using this dosing schedule shows it to have reached

70% of its maximum activity at this time (Fig. 5). A dose of 30 $mg.kg^{-1}$ was initially used but since no discrimination was apparent after 15 sessions it was increased to 50 $mg.kg^{-1}$. This dose was subsequently used in all experiments except those where the effect of training dose on sessions to criterion were being studied. For these experiments doses of 50, 75, 100 and 200 $mg.kg^{-1}$ were used. Rats received drug or saline in each session according to a double alternation sequence which ensured that each drug training day was immediately followed by an equal number of drug and saline training days. To control for position preferences of the rats, half were trained to turn left and half right after saline, with the opposite direction choice being required after 5-HTP. During one experiment the occurence of vicarious trial and error (VTE) was recorded. This was defined as occasions when the rat stopped at the choice point and looked down each side arm before making its choice. The use of the term vicarious refers to the impression that the rat is going down each side arm in its mind to work out the outcome of either choice.

Training continued until the rats reached a criterion of eight correct first trial responses out of ten consecutive sessions, when they were subsequently used for transfer and antagonism studies. These experiments were carried out on Tuesdays and Fridays with training continuing on other days to give both a continuous record of the animals performance and to provide a constant source of reference states for them to compare the experimental condition with. For each experiment the effect of the previous days training on an animals performance was kept to a minimum by having an equal number of animals in each experimental group that received saline and 5-HTP

on the day before. While this was not always possible, any bias so introduced was kept to a minimum. On experimental days both safe areas were available to the rats and only one trial was carried out to avoid reinforcing incorrect responses.

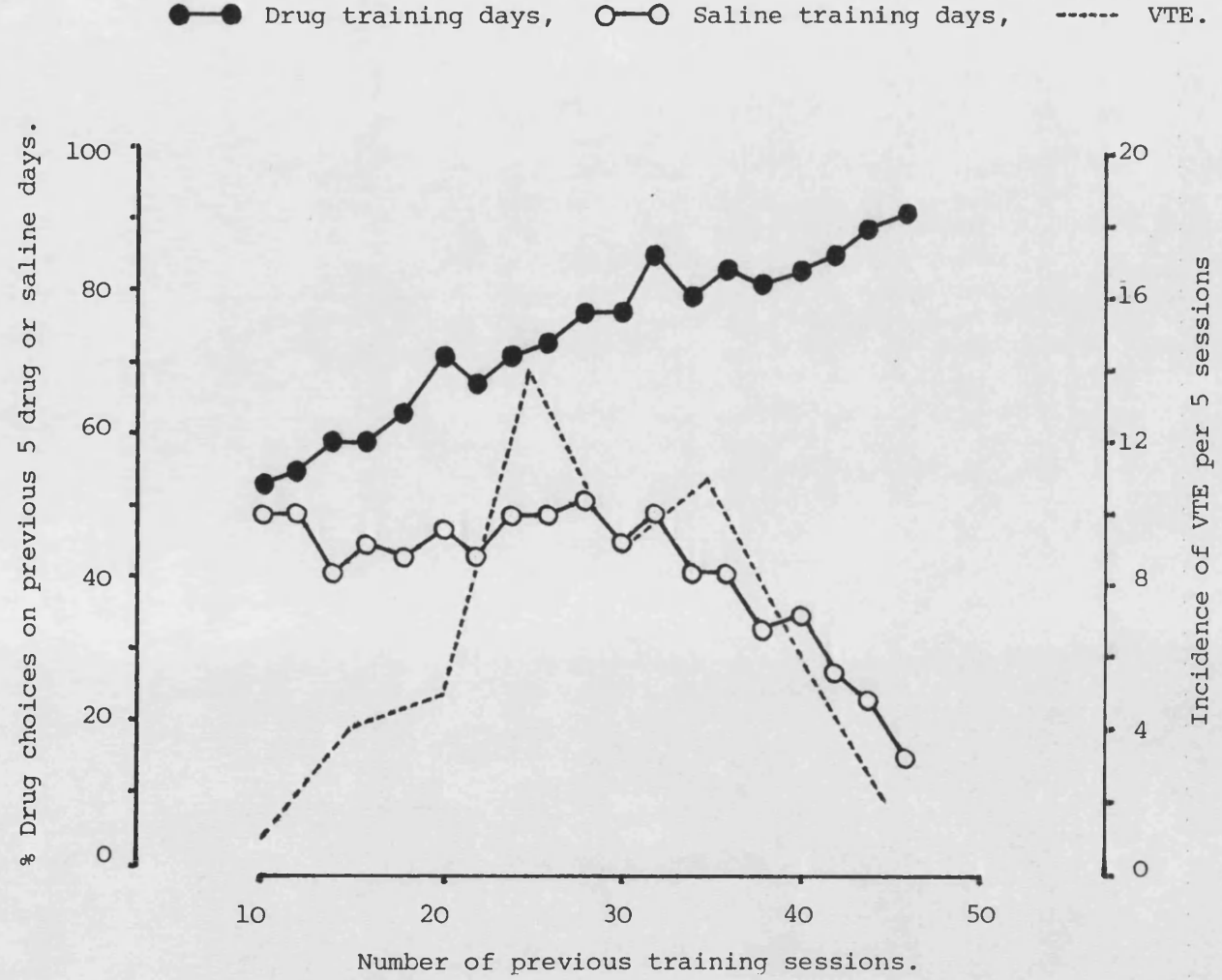
The following agents were tested for their ability to transfer to the 5-HTP cue. The time of administration before testing in the maze is given in brackets: 5-HTP (as on training days); 5-MeODMT (10 min.); RU 24969 (30 min.); quipazine (30 min.); 8-OHDPAT (30 min.); TFMPP (15 min.) and amphetamine (30min.) . For antagonism studies rats received carbidopa and 5-HTP as on a usual drug training day but were tested in the maze in the presence of an antagonist. The antagonists used were: pirenperone (60 min.) pindolol (60 min.) and quipazine (30 min.).

2.3.4 Acquisition of discrimination

Throughout the series of experiments studying the cue properties of 5-HTP, 8 out of 106 rats failed to learn the discrimination within 50 sessions. The remainder learned the task within 15 to 45 sessions, a mean value of 23.8 $\pm$ 2.1 sessions (mean $\pm$ s.e.m.) being obtained for one group of 8 rats which was fairly typical, although one group of 9 took 32.6 $\pm$ 3.5 sessions.

The acquisition of discrimination for one group of rats is shown in Fig. 11. It was a repeated finding that the performance on saline training days was substantially worse than on drug training days, and was often well below the 50% level expected by chance. Also shown on this graph is the number of times each week that the rats exhibited VTE. Despite the fact that sudden changes will be hidden by the averaging of the group of rats it is clear that this

Figure 11. Aquisition of discrimination between saline and 5-HTP in a group of 10 rats.



feature of maze behaviour rises to a maximum about one week before the average time taken to reach criterion and rapidly falls off after this time as the rats become more certain about the discrimination task.

The effect of training dose on the number of sessions needed for the rats to reach criterion was clearly demonstrated, as shown in Table 2. Those trained on the highest dose learned the task significantly faster than those on the lowest, while those on an intermediate dose learnt at an intermediate rate. The top dose of 200 $mg.kg^{-1}$ was abandoned as the onset of symptoms of the 5-HT syndrome disrupted the ability of the animals to perform in the maze.

Table 2. The effect of training dose on the rate of aquisition of discrimination of 5-HTP and saline.

Training dose ($mg.kg^{-1}$)	n	Number of sessions to criterion (mean ± s.e.m.)
50	8	23.8 ± 2.1
75	7	20.0 ± 1.3
100	8	14.0 ± 1.3

2.3.5 Results of transfer and antagonism experiments.

The dose dependent nature of the 5-HTP cue, already demonstrated by varying the training dose, was also shown by testing various doses of 5-HTP on rats trained to discriminate 50 $mg.kg^{-1}$ from saline. As the dose was lowered, a progressively higher proportion of the rats behaved as if they had received saline prior to testing in the maze, until doses of 4.4 $mg.kg^{-1}$ and below were indistinguishable from saline pretreatment (Fig. 12).

The ability of other 5-HT agonists to transfer to the 5-HTP cue is shown in Figs. 13 and 14. Only 8-OHDPAT fails to transfer completely, but at doses of 2 $mg.kg^{-1}$ and above the hyperactivity and fore-paw treading induced by this compound appeared to interfere with the animal's performance. Another factor may be the hyperalgesia that it has been reported to induce (Fozard and Tricklebank, 1983) which would make the foot-shock far more aversive than normal and could lead to random "panic responding".

The effects of other agents tested in the discrimination task, which failed to transfer to the 5-HTP stimulus are shown in Table 3.

Table 3. Compounds failing to transfer to the 5-HTP cue.

Drug	Dose ($mg.kg^{-1}$)	n	% choosing drug direction
Quipazine	2.5	18	11
Amphetamine	0.5	8	12.5

Figure 12. Dose-response curve for transfer to 5-HTP in rats trained to discriminate 5-HTP (50 mg/kg) from saline.

Numbers in brackets refer to the number of rats tested at each dose.

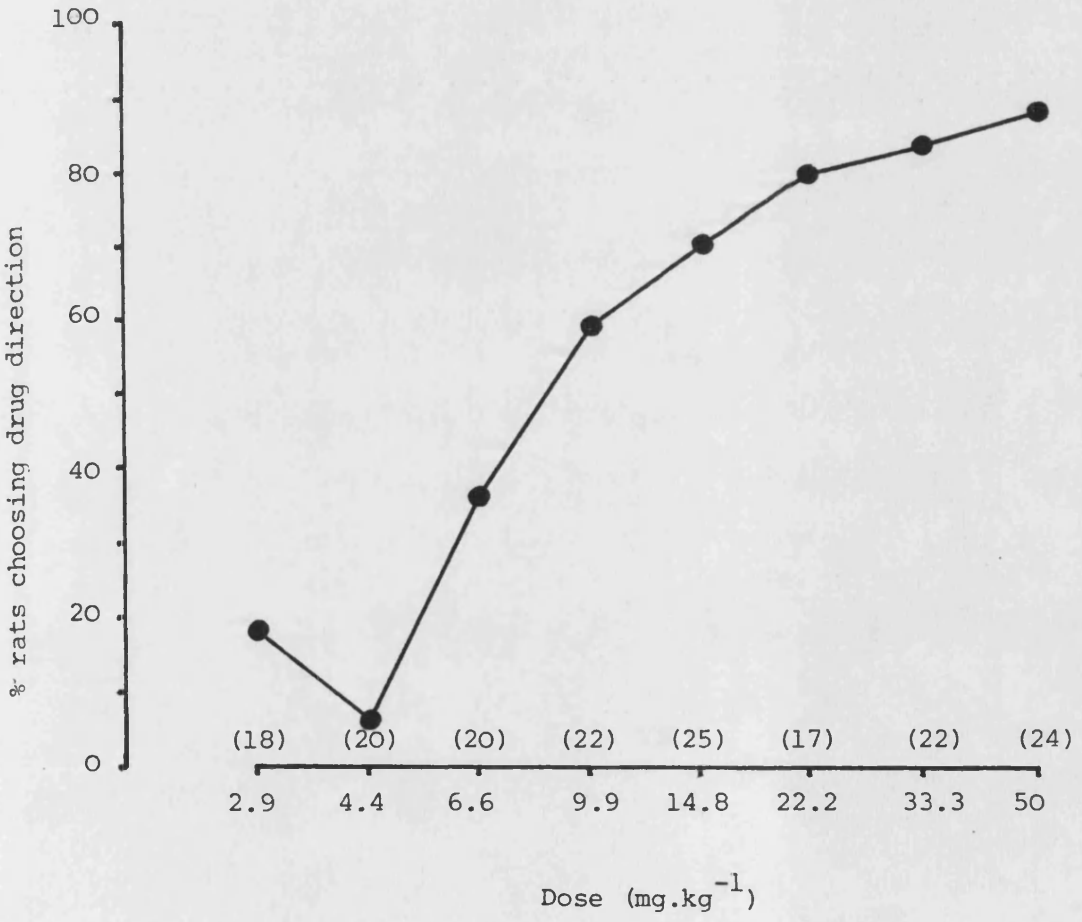


Figure 13. Transfer of 5-HT agonists to the 5-HTP discriminative stimulus.

Numbers in brackets refer to the number of rats tested at each dose.

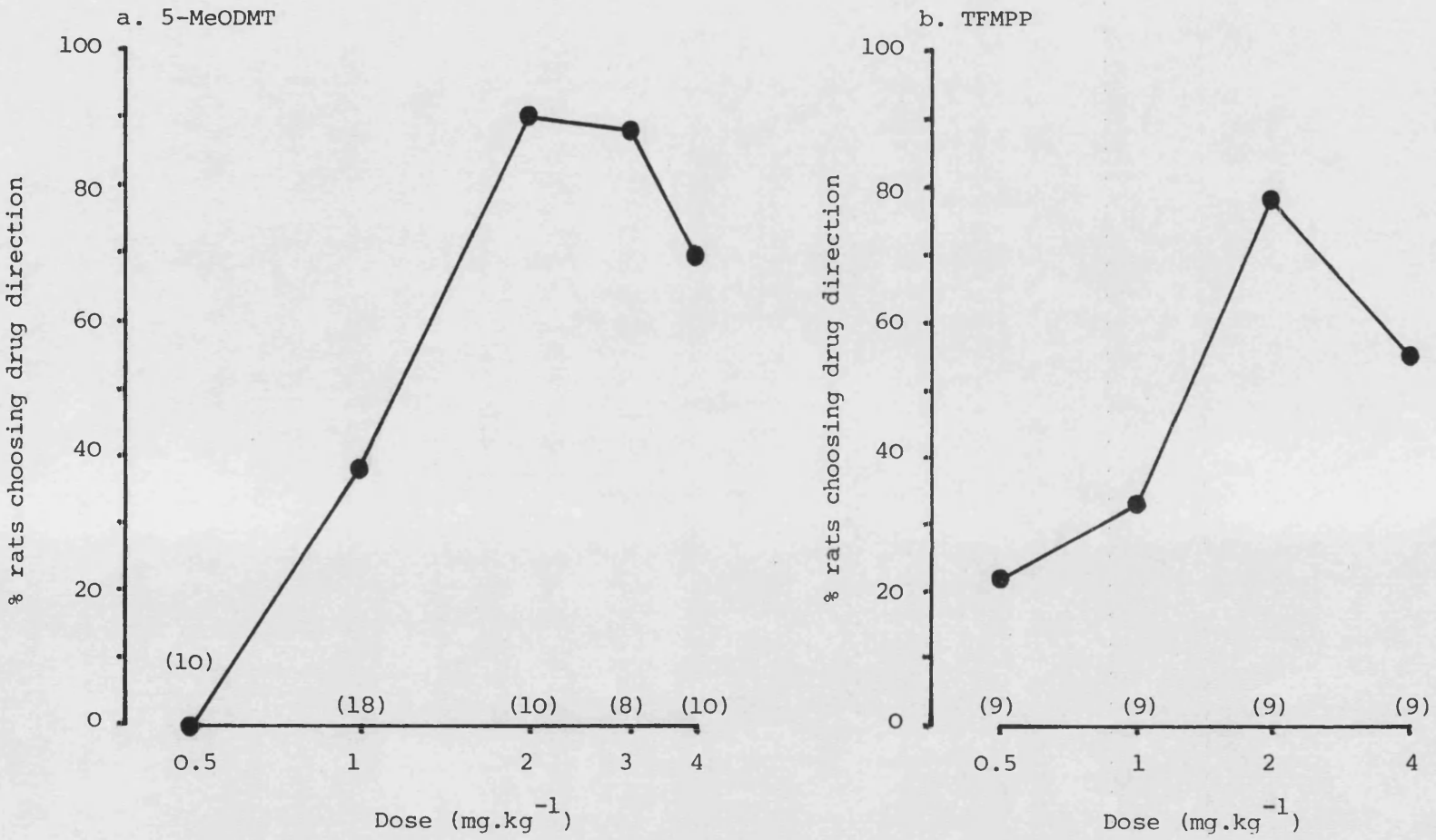
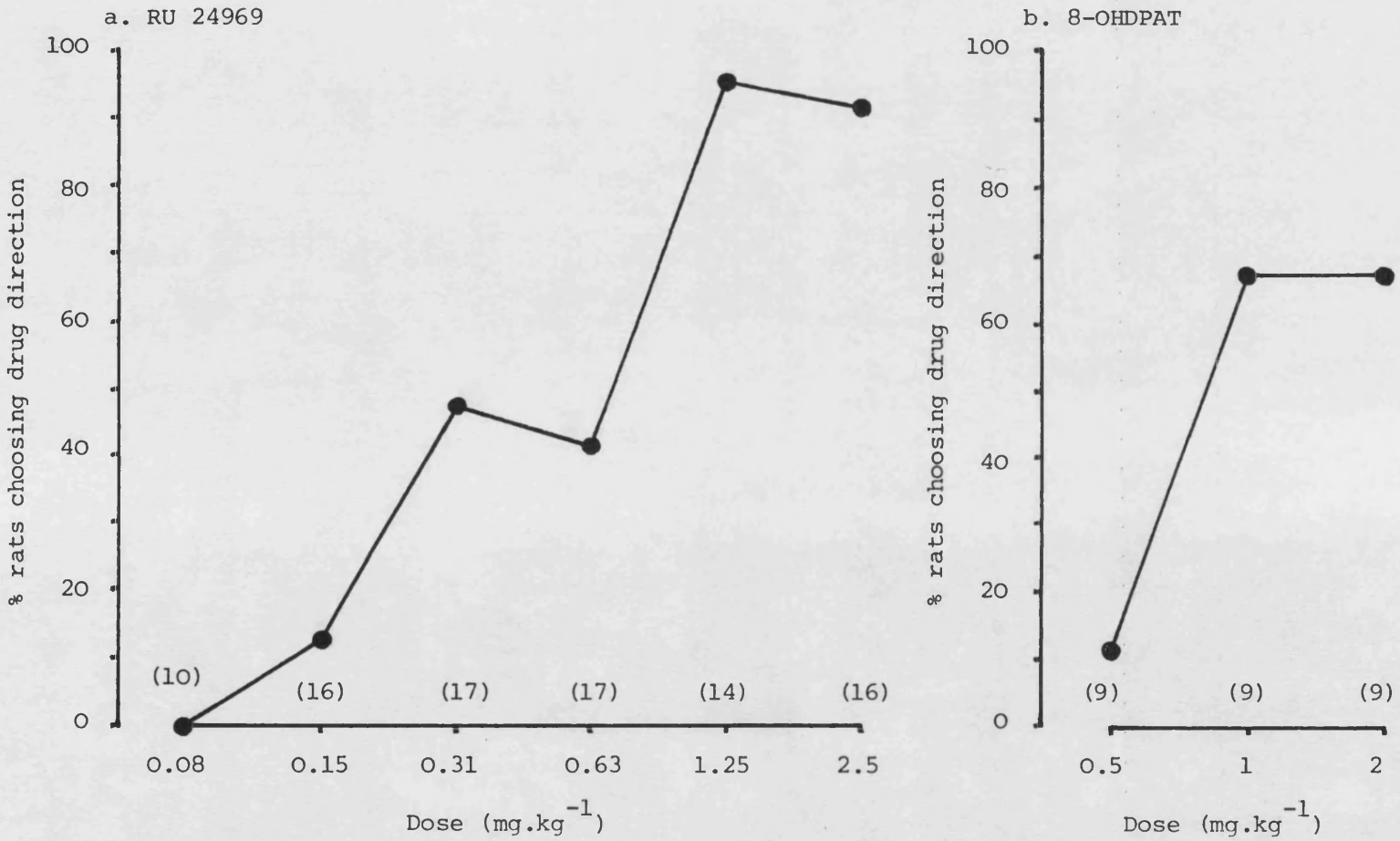


Figure 14. Transfer of putative 5-HT_1 agonists to the 5-HTP discriminative stimulus.

Numbers in brackets refer to the number of rats tested at each dose.



Two antagonists, pirenperone and pindolol, were studied for their effects on the 5-HTP cue. Pirenperone was found to be completely ineffective as an antagonist of this property of 5-HTP at doses up to 400 $\mu g.kg^{-1}$, with none of the 8 animals tested turning in the saline direction. It should be remembered that its ED_{50} for inhibition of 5-HTP induced head-twitches was only 2.3 $\mu g.kg^{-1}$ using the same dosing schedule as for the discrimination study and against a higher dose of 5-HTP (Fig. 9). Pindolol was also ineffective against the 5-HTP cue at doses upto 8 $mg.kg^{-1}$. At this dose none of the 9 animals tested turned in the saline direction. Quipazine has been shown to act as an antagonist at the presynaptic 5-HT receptor, but it too had no effect on the 5-HTP cue as none of the 5 animals tested at 2.5 $mg.kg^{-1}$ turned in the saline direction.

2.4 RU 24969 INDUCED HYPERACTIVITY

2.4.1 Introduction

Since the evidence for multiple 5-HT receptors in the CNS was first published there has been a search for drugs which would act preferentially upon one or the other of these receptors. The first to be produced were the 5-HT_2 antagonists ketanserin, pirenperone and more recently, ritanserin. The first drug which was claimed to have selectivity for the 5-HT_1 site was RU 24969 which was shown to be a potent displacer of ligands for this site (Hunt et al., 1981; Hunt and Oberlander, 1981). This putative agonist was found to have a different profile of behavioural activity to other agents acting as 5-HT agonists. When given to mice it induced a long lasting, continuous hyperactivity and at no time were any head-twitches observed (Gardner and Guy, 1983). The antagonist methergoline inhibited the hyperactivity but propranolol which has been shown to have 5-HT_1 antagonist properties (Nahorski and Willcocks, 1983) only weakly reversed it.

The status of RU 24969 hyperactivity as a 5-HT_1 mediated effect is therefore not entirely unequivocal, as recently suggested by Tricklebank (1984a) and as discussed in section 1.4, but this may be due more to the lack of 'custom-built' antagonists rather than the actions of the drug itself. It is however, the only drug currently available with selectivity for the 5-HT_{1B} site, and further examination of the consequences of stimulation of this receptor will require the production of a full range of agonists and antagonists for the various 5-HT receptor subtypes. The recent suggestion by Asarch et al. (1985) that the 5-HT_{1B} binding site shows signs of

being a heterogenous population may lead to a better understanding of these results. Another aspect that is bound to make interpretation difficult is that while the hyperactivity may be initiated by 5-HT receptors it is almost certainly expressed via a catecholaminergic system (Green and Heal, 1985).

2.4.2 Methods

The hyperactivity induced by RU 24969 was studied in the mouse using the open-field apparatus. The arena was 1m in diameter with 30cm high side walls. The floor area was divided into 16 areas by means of 8 equally spaced radial lines and an 80cm diameter circle drawn on the base. Mice were dosed with RU 24969 and 30 min later were placed in the centre of the open-field. The mice were observed and the number of line crossings in the following 2 min period was counted.

The effects of the 5-HT_2 antagonist pirenperone and the 5-HT_1 antagonist (-)-pindolol and its isomer (+)-pindolol were studied on the hyperactivity induced by 1.25 $mg.kg^{-1}$ RU 24969. The antagonists were given 30 min before RU 24969.

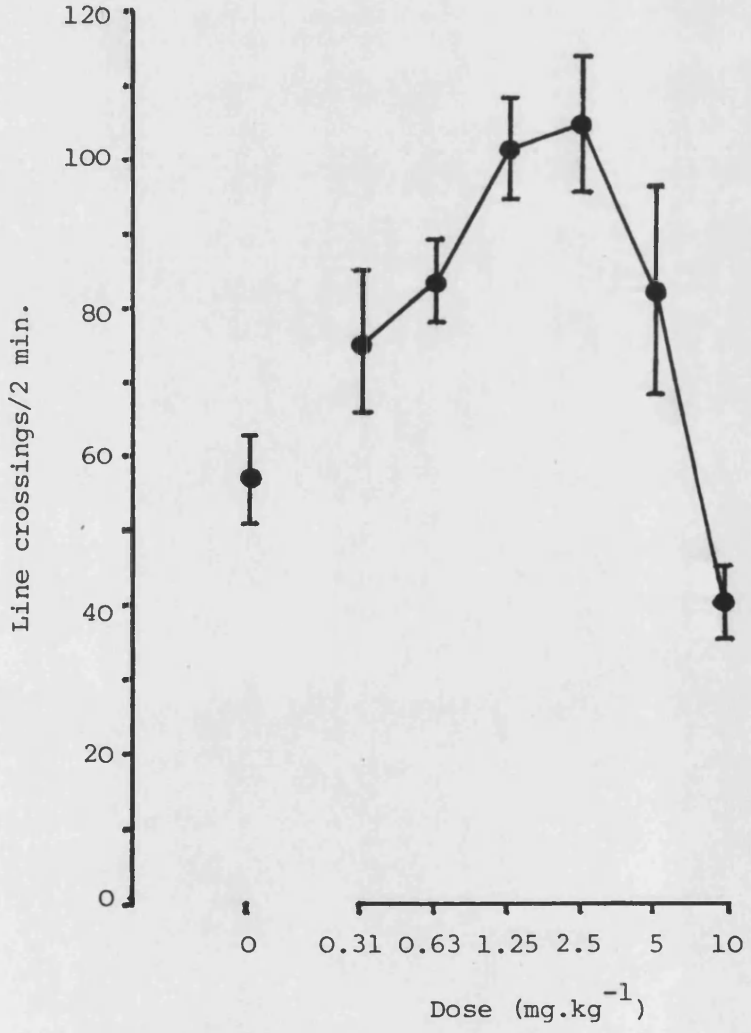
2.4.3 Results

The dose-response relationship for RU 24969 hyperactivity in the open-field is shown in Fig. 12. It can be seen that the response is bell-shaped, rapidly falling off after the peak effect at 2.5 $mg.kg^{-1}$. At the highest dose used all the mice exhibited a degree of tremor and hind-limb abduction. This was also observed in 50% of those mice which received 5 $mg.kg^{-1}$.

The effects of the antagonists studied are shown in Table 4. No

Figure 15. Effect of RU 24969 on locomotor activity in the open field in mice.

All values are mean ± s.e.m., n=6.



inhibition was seen with pirenperone while both isomers of pindolol only slightly inhibited the response. None of the differences reached significance using Student's t-test.

Table 4 Inhibition of RU 24969 hyperactivity by 5-HT antagonists

Treatment	Dose ($mg.kg^{-1}$)	n	Line-crossing (mean ± s.e.m.)	% inhibition (mean ± s.e.m.)
Saline	-	6	56.0 ± 5.1	
RU 24969	1.25	6	108.0 ± 5.3	
RU 24969 + Pirenperone	1.25 + 5 $\mu g.kg^{-1}$	6	114.2 ± 6.4	-11.9 ± 12.3
RU 24969 + (-)-Pindolol	1.25 + 4	6	97.5 ± 5.7	28.3 ± 15.0
RU 24969 + (+)-Pindolol	1.25 + 4	6	93.3 ± 7.8	20.2 ± 11.0

2.5 8-OHDPAT INDUCED HYPOTHERMIA

2.5.1 Introduction

Another recently introduced compound which shows many properties consistent with being a potent 5-HT agonist is 8-OHDPAT. It has been shown to decrease 5-HT synthesis and also to induce some of the signs of the 5-HT syndrome (Hjorth et al., 1982). On the basis of ligand binding studies Middlemiss and Fozard (1983) have suggested that 8-OHDPAT is selective for the 5-HT_{1A} receptor, and further work by Tricklebank (1984c) indicates that the fore-paw treading induced by 8-OHDPAT is a result of activation of these receptors, as it is antagonised by (-)-pindolol and by spiperone (which discriminates between the two 5-HT_1 sites) but not by ketanserin. It is interesting that the 5-HT syndrome has been reported to be antagonised by 5-HT_2 antagonists when induced by quipazine or 5-MeODMT but not when induced by 8-OHDPAT (Tricklebank et al., 1984 ; Goodwin and Green, 1983). It has been suggested that this indicates that there is a 5-HT_{1A} link between the 5-HT_2 receptors and the final pathways mediating the response (Goodwin and Green, 1985), but clearly more work is needed on this aspect of the pharmacology of 8-OHDPAT.

In rats, 8-OHDPAT induces a dose related hypothermia which is stereoselectively antagonised by (-)-pindolol and (-)-propranolol (Middlemiss et al., 1985; Goodwin and Green, 1985). Antagonists of the 5-HT_2 receptor do not affect 8-OHDPAT-induced hypothermia and neither do β-adrenoceptor antagonists which show no affinity for 5-HT receptor sites.

All the experiments described above were performed in the rat but preliminary studies showed that 8-OHDPAT could also induce

hypothermia in the mouse. It was consequently decided to use this species to complement the work on the head-twitch response in the mouse. Since these studies were performed a detailed analysis of the 8-OHDPAT induced hypothermia in mice has been reported by Goodwin et al., (1985) which strongly suggests that this response is mediated presynaptically, as it is abolished by lesions of the presynaptic 5-HT terminals and antagonised by quipazine which has been shown to have presynaptic antagonist properties (Martin and Sanders-Bush, 1982). Goodwin et al., (1985) have also cast doubt on the idea that this response is 5-HT_{1A} mediated as (-)-pindolol and (-)-propranolol were without effect. In the light of these results it is difficult to positively ascribe a receptor type to this response but the evidence available would indicate that the 5-HT_{1A} subtype is the most likely. Reliance on the results from antagonists that have been characterised using the rat may be the source of confusion as it seems that responses in these two species to 5-HT agonists show many differences. Hjorth and Carlsson (1985) have recently provided evidence that (-)-pindolol may act as an agonist at 5-HT autoreceptors. This observation may help to solve some of the previous discrepancies.

2.5.2 Methods

Mice were given various doses of 8-OHDPAT s.c. immediately following the measurement of their rectal temperature by insertion of a temperature probe 2cm into the rectum. After a 30 min period the rectal temperature was again measured. All measurements were carried out at an ambient temperature of 20-23^{o}C.

The effects of the 5-HT antagonists pirenperone and pindolol

were examined on the hypothermia induced by 2 $mg.kg^{-1}$. The antagonists were given 30 min before 8-OHDPAT and the rectal temperature was also measured before the administration of the antagonists.

2.5.3 Results

8-OHDPAT was found to produce a dose related fall in rectal temperature in mice with a maximum fall in body temperature of 2.5°C being obtained at doses of 1 $mg.kg^{-1}$ and above (Fig. 13).

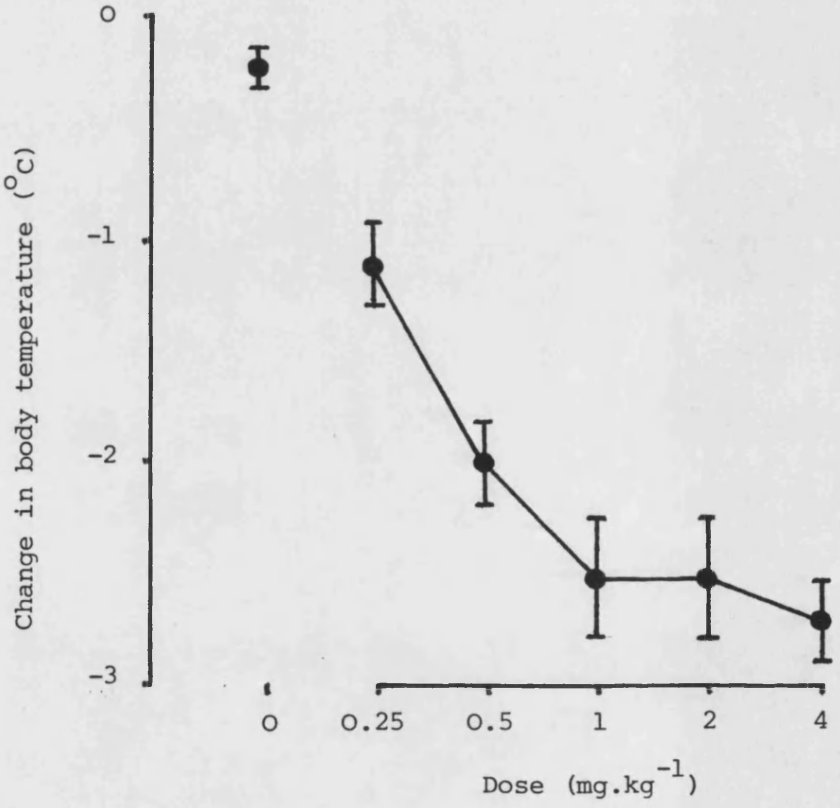
The effects of the antagonists are shown in Table 5. In agreement with previous reports pirenperone was completely without effect but, in contrast to earlier data there was some evidence of inhibition with pindolol. This inhibition was found to be stereo-selective, with the (-) isomer being a lot more potent, a result consistent with the mediation of this response by the $5\text{-}HT_{1A}$ receptor. None of the antagonists induced a temperature change when given alone.

Table 5 Effect of antagonists on 8-OHDPAT induced hypothermia

Drug	Dose	n	% inhibition (mean ± s.e.m.)
Pirenperone	$5ug.kg^{-1}$	8	0.0 ± 13.6
(-)-Pindolol	$4mg.kg^{-1}$	8	40.9 ± 9.0
(+)-Pindolol	$4mg.kg^{-1}$	8	13.6 ± 13.6

Figure 16. Effect of 8-OHDPAT on body temperature in mice.

All values are mean ± s.e.m., n=8.



2.6 DISCUSSION

Of the methods described in this section, the 5-HT agonist-induced head-twitch is the most established method, with a large amount of data supporting its mediation by the 5-HT_2 receptor subtype. This has already been discussed in Section 1.4 and will not be repeated here, but two recent reviews by Green and Heal (1985) and Goodwin and Green (1985) both conclude that the head-twitch response in both rats and mice is a good model for the 5-HT_2 receptor. The data presented here fully support this contention as the response is induced by a range of 5-HT_2 agonists which have varying effects on other 5-HT receptors and it is antagonised by the selective 5-HT_2 antagonist pirenperone but not by the putative 5-HT_1 antagonist (-)-pindolol.

However, the picture is not quite as clear as this. All available evidence indicates that 5-MeODMT is an agonist at both 5-HT_1 and 5-HT_2 receptors (see Section 2.2.1) but it was not found to induce head-twitches in the rat. A possible explanation for this is that the presence of a severe 5-HT syndrome hides the head-twitches by means of some sort of "behavioural competition" whereby it is not physically possible for the rat to display both behaviours at once. It must be said, however, that this was not found to occur in mice, which may point to a more fundamental difference in the properties of 5-HT receptors, or of behaviours mediated through them, in these two species. This point seems to be born out by a study of the pharmacology of putative 5-HT_1 mediated effects which show many differences between rats and mice as indicated in the introductions to the various methods described in this section. As suggested in many published reports, the unravelling of these

problems requires the development of selective agonists and antagonists for the receptor subtypes, and perhaps more reliance on drug responses rather than binding data if we are not to fall into similar problems to those encountered in the field of dopamine receptor research (Green, 1985).

In common with the findings of Lucki et al. (1984), the results obtained using the 5-HT syndrome are consistent with it being mediated by a 5-HT receptor that is different to that mediating the head-twitch. The observation that 5-MeODMT will predominantly induce the syndrome, while 5-HTP will predominantly induce head-twitches, would support this, as does data from antagonist studies showing that pirenperone is a far more potent antagonist of the head-twitch than it is of the syndrome. As the inhibition of the 5-HT syndrome by pirenperone was only seen at very high doses, this may not even be due to an action at 5-HT receptors. The fact that (-)-pindolol was not found to antagonise the syndrome in mice might indicate that the 5-HT_1 receptor is not involved, but many results with this compound seem inconsistent (see previous sections) and this observation should perhaps be treated with caution.

The use of the syndrome as a measure of central 5-HT receptor activity, as applied to 5-HT receptor subtypes, has not been fully explored, possibly due to its complicated nature, its reliance on multiple symptoms and the involvement of other neurotransmitters (Tricklebank et al., 1985). The examination of individual components such as fore-paw treading has already proved more fruitful (Tricklebank, 1984b, 1985) and it is likely that the scoring system used in this thesis is not sophisticated enough to make a proper study of these behaviours possible. A report by Dickinson et al., (1983)

has shown that the scoring system used in the evaluation of the syndrome can affect the conclusions to be drawn from an experiment and the use of a 3 or 4 point scale may not be enough to show up differences that are in fact there.

It was this problem that necessitated the use of other models of 5-HT_1 receptor activation in order to study this receptor over 24 hours. The behaviours induced by the recently described 5-HT agonists 8-OHDPAT and RU 24969, introduced on the basis of their ligand binding properties, have perhaps provided useful alternatives to the syndrome. Even their properties however, are not without controversy, as not all the whole animal data is consistent with the binding data. The results given here with pirenperone support the widely held view that the effects of these agents are not mediated by the 5-HT_2 receptor (e.g. Goodwin and Green, 1985) but it is not possible to be certain about the exact identity of the receptor involved. If the definition of the 5-HT_{1A} receptor is that it is stimulated by 8-OHDPAT and antagonised by (-)-pindolol (Middlemiss et al., 1985) then the data from these experiments would suggest that 8-OHDPAT-induced hypothermia in the mouse is mediated by this receptor subtype, even if it is located presynaptically as suggested by Goodwin et al., (1985). It should be noted that Goodwin et al., (1985) obtained no antagonism of this effect in mice with (±)-pindolol or with (-)-propranolol and that the stereoselective antagonism found in these experiments with pindolol required high doses which only reversed the hypothermia by less than 50%. It is possible that at these doses the moderate selectivity of (-)-pindolol for the 5-HT_{1A} site over the 5-HT_{1B} site no longer exists (Middlemiss et al., 1985).

The results with RU 24969 would indicate that neither the 5-HT_2 nor the 5-HT_{1A} receptors are involved, and, if (-)-pindolol is not selective at the dose used, then the 5-HT_{1B} recptor might not be involved either. Without better evidence it has to be assumed that the 5-HT_{1B} receptor is responsible for the hyperactivity response to RU 24969, although this may prove to be erroneous when better drugs are available to study it.

The data obtained using the 5-HTP discriminative stimulus has added an interesting twist to the distinction between 5-HT_{1A} and 5-HT_{1B} receptor sites as both 8-OHDPAT and RU 24969 will generalise to it, indicating that they have a common pharmacological property. The 5-HTP cue has clearly been shown not to be mediated by the 5-HT_2 receptor as pirenperone had no effect at doses well in excess of those necessary to inhibit completely the head-twitch response in rats induced by a dose of 5-HTP higher than the cue dose. The absence of transfer to quipazine at a dose that has been shown to have cue properties via the 5-HT_2 receptor (Friedman et al., 1984) also supports the lack of involvement of the 5-HT_2 receptor. These results thus support and extend the observations of Barrett et al. (1982) and Friedman et al. (1983). Evidence that the 5-HT_1 receptor is involved comes from the full transfer to the 5-HTP cue of RU 24969 and of TFMPP. These two drugs both show more selectivity for the 5-HT_1 binding site than the 5-HT_2 site (Martin and Sanders-Bush, 1982; Hunt and Oberlander, 1981) and TFMPP has itself recently been used as a discriminative stimulus (Glennon et al., 1984) which also transfers to RU 24969 but not so well to 5-HT_2 agonists such as DOM (1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane) (Shannon et al., 1985). The effect of 8-OHDPAT, which shows signs of transfer

before disruptive effects occur, might also indicate that the 5-HT_1 receptor is involved, although the effect observed could be due to random responding. The lack of antagonism by the 5-HT_{1A} antagonist (-)-pindolol suggests that the latter is more likely, and that this receptor subtype is not involved in this process. The lack of antagonism by quipazine, which can act as a presynaptic 5-HT antagonist (Schlicker and Gothert, 1981), indicates that the response is not mediated by presynaptic 5-HT receptors either. This makes it very difficult to classify the 5-HT receptors involved in this response, but they show similarities with those described by Blackburn et al., (1984) which mediate the rotational behaviour in the unilaterally 5,7-dihydroxytryptamine lesioned rat. Blackburn et al. (1984) did not test (-)-pindolol but did find that both 8-OHDPAT and RU 24969 acted as agonists in this model. Unfortunately they could antagonise these effects with methysergide, which was reported to have no effect against the 5-HTP cue by Barrett et al. (1982).

Of all the putative measures of 5-HT_1 receptor activity discussed above, the 5-HTP cue is perhaps the best, as there is more evidence that it is mediated by a 5-HT receptor (see section 2.3.2), and discriminative control is dose dependent to very low doses of 5-HTP, making it unlikely that release of other neutotransmitters is involved in the response (Ng et al., 1972; Butcher et al, 1972). Also, it is not dependent on the induction of abnormal behaviours or of interference with, or excessive stimulation of normal ones, such as hypothermia or locomotor activity. What evidence is currently available would suggest that responses to RU 24969 or 8-OHDPAT are also mediated by a 5-HT receptor, and certainly their transfer to

the 5-HTP cue would support this.

In view of the current confusion surrounding the classification of behaviours mediated by subtypes of the 5-HT receptor, all of the methods described in this section were used to study the 24 hour variation of 5-HT receptor mediated behaviours.

3 24-HOUR VARIATION OF BEHAVIOURAL RESPONSES TO 5-HT AGONISTS

3.1 INTRODUCTION

Apart from a few reports, the vast bulk of work examining the circadian variation of 5-HT function has dealt with changes in presynaptic function, and our knowledge of this area has become increasingly detailed (see Section 1.2). The final outcome of these effects appears to be that 5-HT neurones release more 5-HT during the dark phase of the light-dark cycle than during the light phase. The importance of this variation, however, is entirely dependent on how this change of activity is transmitted across the synapse, and the lack of previous work on circadian variation of postsynaptic 5-HT mechanisms is therefore surprising. The work of Singleton and Marsden (1981) showed that the head-twitch response to 5-MeODMT in mice varied with time of testing, but the lack of detailed information meant that it was not possible to determine the position of highest and lowest activity in relation to the light-dark cycle. This data is essential if we are to understand how pre- and postsynaptic functions are integrated over 24 hours.

In the experiments described in this section a variety of behaviours that involve 5-HT receptors for their initiation were studied over a 24 hour period so that a better understanding of how 5-HT functional activity changes over 24 hours will be obtained. The behaviours used have been discussed in the previous chapter, and while there is some doubt about the nature of the 5-HT receptor involved in some of them, there is sufficient evidence available to support the belief that a 5-HT receptor is involved (see also Section 1.3).

3.1.1 General considerations

In the following experiments, the 24-hour variation of various drug-induced behaviours was studied by performing identical experiments at equally spaced intervals throughout the light-dark cycle. For many of the techniques used it would be impractical to study individual animals at exactly the same time point, and in any case some finite time is necessary for drugs to be absorbed etc., and have their effect. Therefore in practice the experiment was performed around the designated time point. The greatest difference between the actual time of the experiment and the designated time was limited to 60 min. and in most cases it was considerably less than this.

It would also be impractical to study animals over a continuous 24 h period, and in order to facilitate measurements in the middle of the dark phase some groups of animals were phase-shifted so that their dark-phase coincided with the light phase of the experimenter. All animals were therefore maintained under controlled conditions that included a 12h light-12h dark cycle using environmental cabinets previously described by Hillier et al., (1973). They were housed under the experimental conditions for at least 14 days before each experiment. Previous experiments at the University of Bath have shown that this period allows animals to fully adapt to the new lighting schedule.

The terms '24-hour' and 'circadian' have been used as equivalent in this thesis. This is in keeping with the way they are currently used in describing experiments of this type, reflecting a belief that the variations observed are endogenously generated.

Animals and drugs were as previously described in Section 2.1.1.

3.2 CIRCADIAN VARIATION IN HEAD-TWITCH AND 5-HT SYNDROME RESPONSE

3.2.1 Methods

The head-twitch response to 5-MeODMT, 5-HTP, quipazine and mescaline, and the syndrome response to 5-MeODMT were studied in mice. The 5-HTP induced head-twich response was also studied in rats. The methods used for each of these drugs was exactly as described in Section 2.2. The doses used for each agonist are given in the results section.

All observations were carried out under normal room lighting. In order to make sure that this was not affecting the results, an experiment was also carried out under dim red light.

The presence of circadian variation was established using analysis of variance. The data for 5-MeODMT induced head-twitches over 24h was also analysed using best-fit cosine curve analysis by Professor F.Halberg, University of Minnesota.

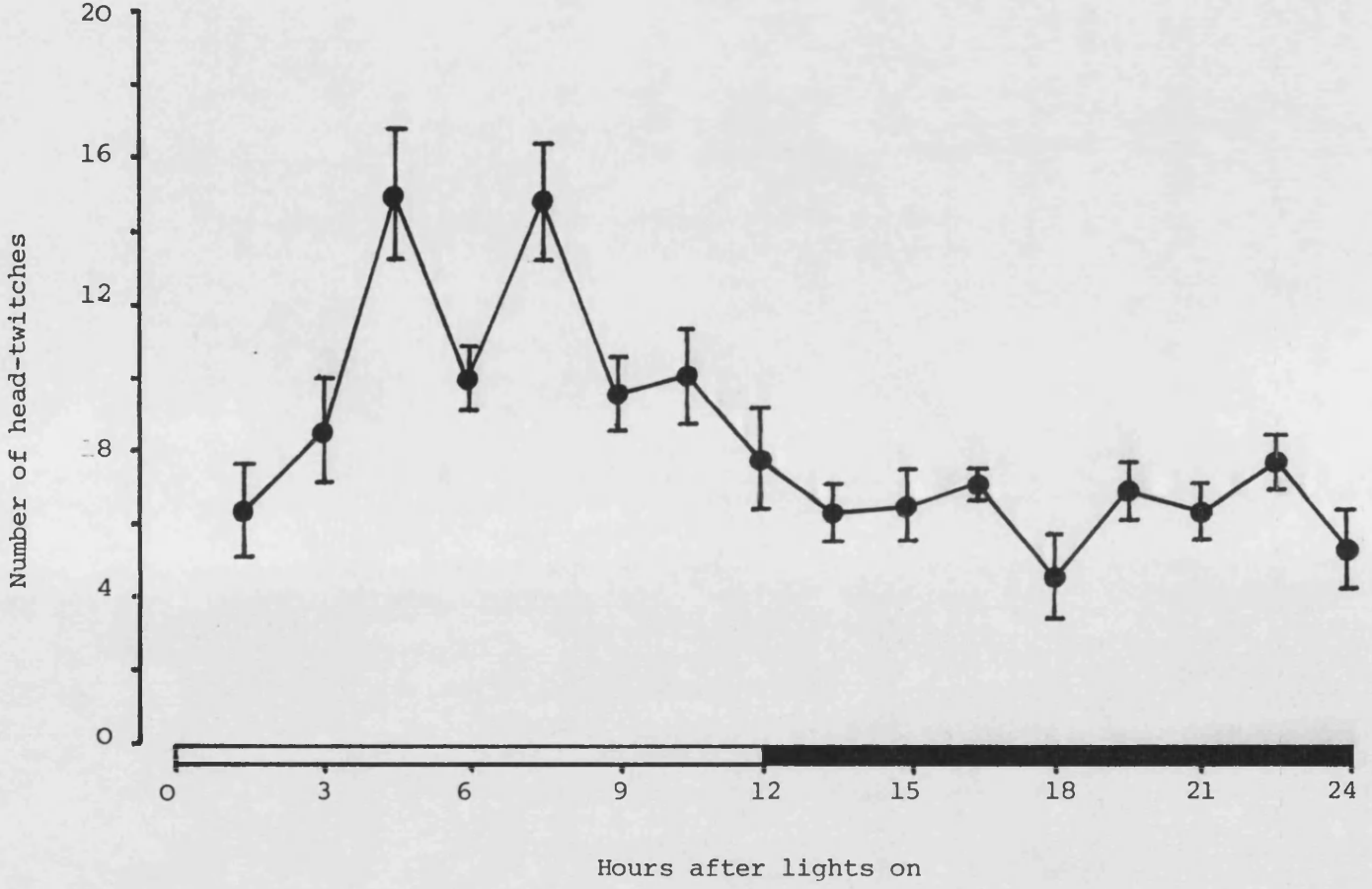
3.2.2 Effect of 5-MeODMT in mice

When different groups of 8 mice were tested at 1.5h intervals throughout the 24h light-dark cycle by injection of 5 $mg.kg^{-1}$ 5-MeODMT, a dose which lies midway on the dose-response curve (Fig. 2), a clear circadian variation in head-twitch response was evident (Fig. 17). Analysis of variance indicated that there was a statistically significant variation between the mean values over 24h ($F=6.4$ for 16/118 DF; $p<0.01$). Although a double peak appears in the raw data shown in Fig. 17, cosine analysis showed that the peak of the best fit cosine curve had an acrophase, or peak value, at -103^{o} relative to lights on. The mean value over 24h was 8.41 head-twitches and the amplitude of the cosine curve was 3.01

Figure 17. 24-hour variation of 5-MeODMT-induced head-twitch response in mice.

Each point represents mean ± s.e.m. (n=8) head-twitches in response to 5 $mg.kg^{-1}$ 5-MeODMT.

Black bar represents hours of darkness.



head-twiches. The cosine analysis also indicated that the rhythm was significant ($p<0.002$).

In contrast to the head-twitch response, no circadian variation was evident in either total scores for the 5-HT syndrome, or for any individual component of it (Fig. 18).

The nature of the time-related variation was further investigated by constructing dose-response curves to 5-MeODMT at mid-light and mid-dark, the points in the light-dark cycle at which respectively the peak and trough of head-twitch response was observed. These results are shown in Figs. 19a and 19b and clearly reinforce the data obtained over 24h. Over the lower part of the dose-range, the head-twitch response shows a significant parallel shift to the right when measured mid-dark as compared to mid-light. It is impossible to be sure whether or not this parallel shift persists at higher doses because of the increasing variability of response and the onset of toxicity above 32 $mg.kg^{-1}$ 5-MeODMT. It would appear however, that the maximum has been reduced at mid-dark compared to mid-light. Again, in contrast to the variation in the head-twitch response, the dose-response curves for the 5-HT syndrome measured at mid-light and mid-dark are identical.

To show that the variation in the number of head-twitches at the two time points was not due to the testing conditions, the experiment shown in Fig. 19a was repeated under dim red light. The results of this experiment (Table 6), indicate that the difference between the responses at mid-light and mid-dark persists. It can also be seen that the values obtained were very similar to those in Fig. 19a.

Figure 18. 24-hour variation of 5-MeODMT-induced 5-HT syndrome in rats.

Each point represents the response of 8 mice to 5 $mg.kg^{-1}$ 5-MeODMT

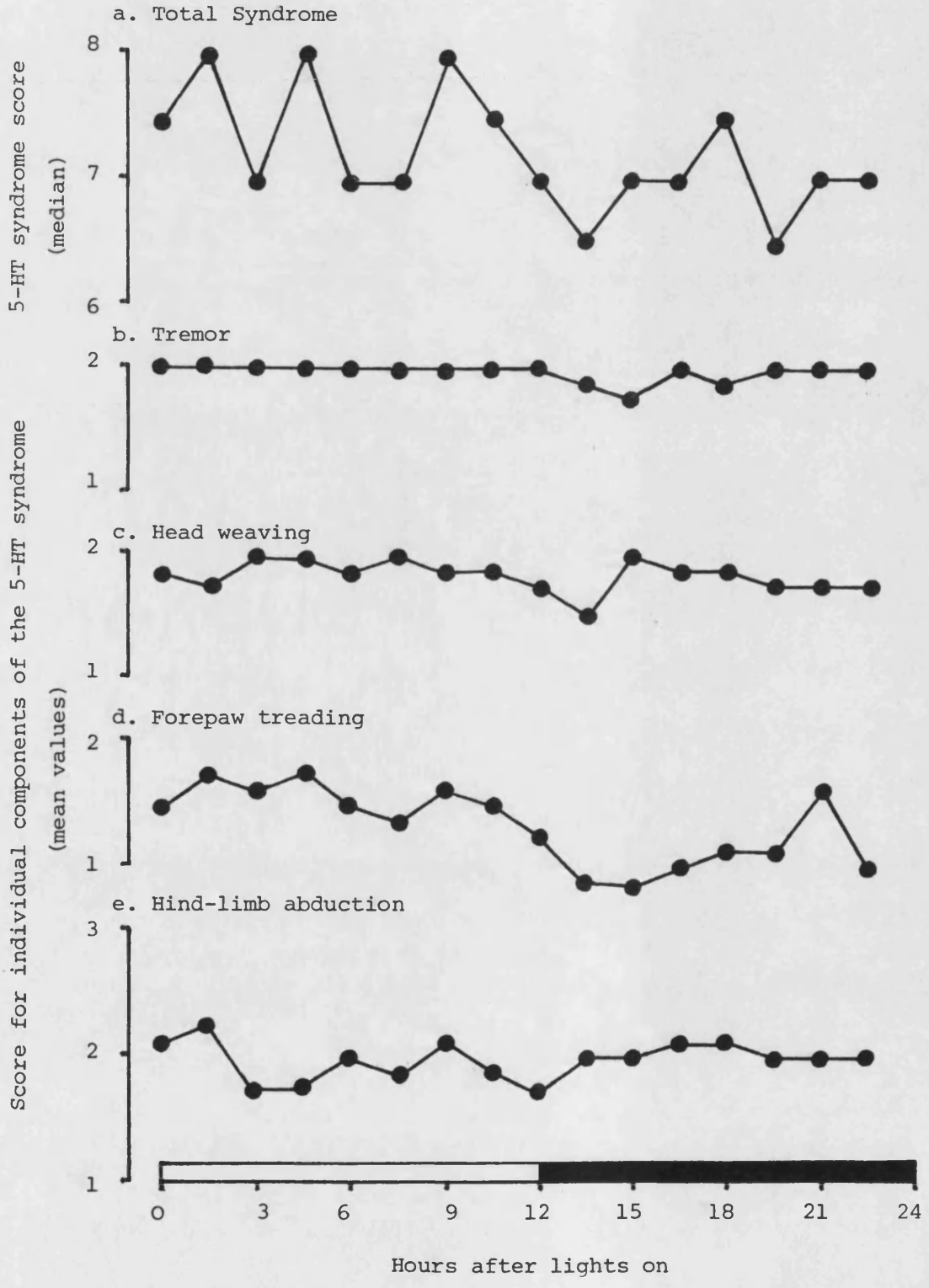


Figure 19. Dose-response curves for 5-MeODMT-induced head-twitches and syndrome at mid-light and mid-dark.

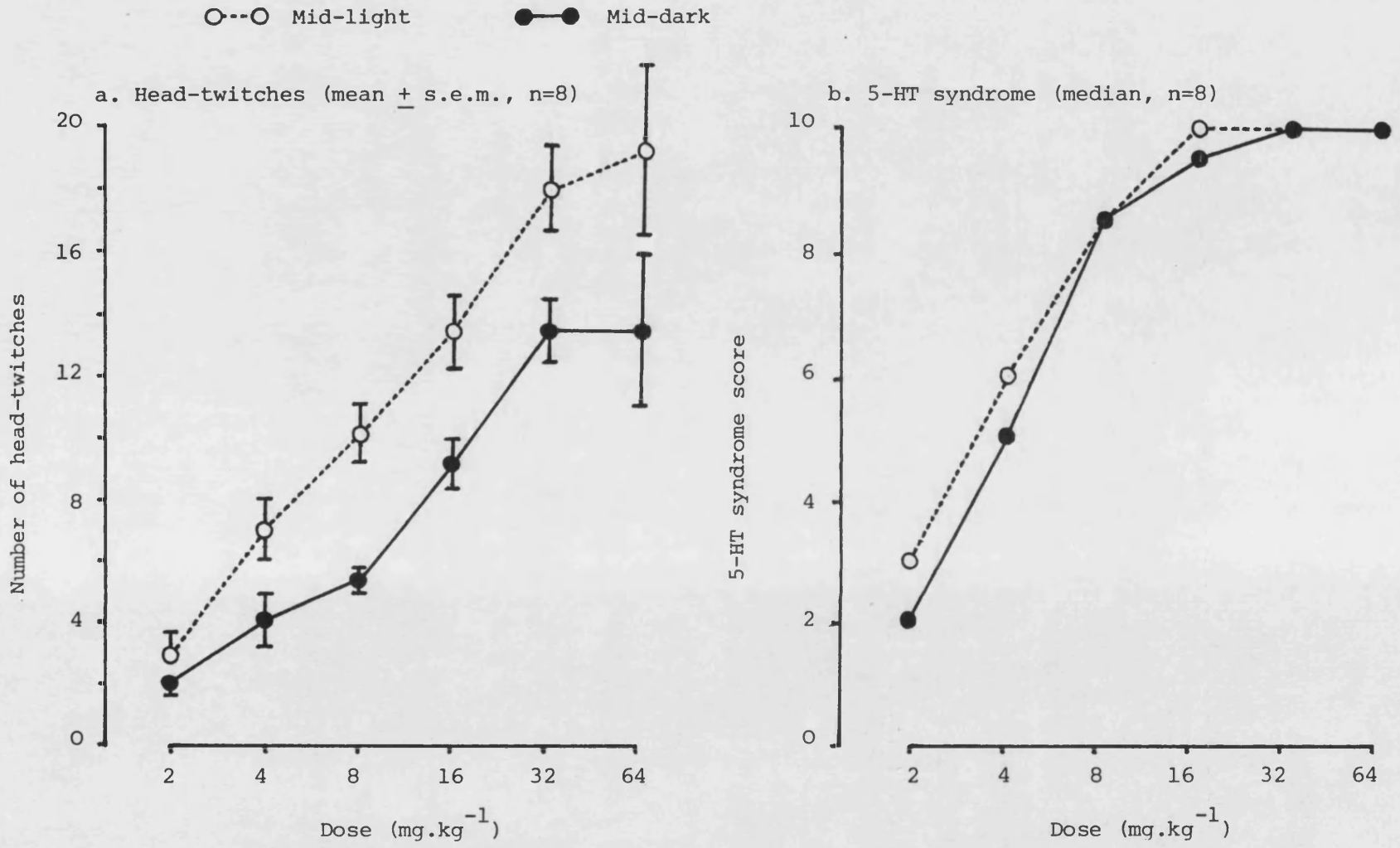


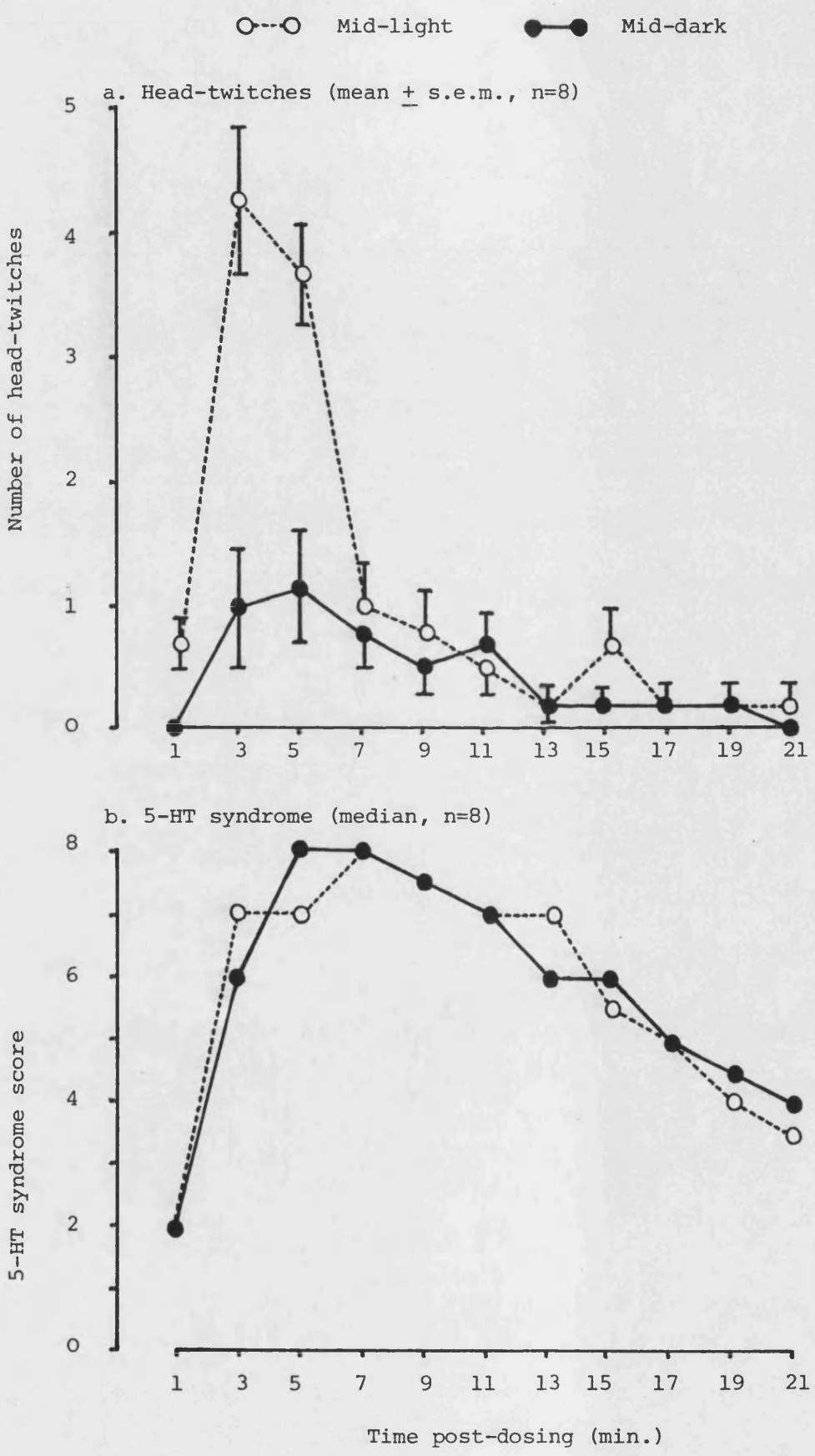
Table 6 Head-twitch response to 5-MeODMT measured under red light

Dose ($mg.kg^{-1}$)	n	Number of head-twitches (mean ± s.e.m.) Mid-light	Mid-dark	t-test
2	8	3.9 ± 0.5	2.4 ± 0.4	$p<0.01$
8	8	12.0 ± 1.1	7.9 ± 0.7	$p<0.01$
32	8	16.6 ± 2.2	12.8 ± 1.3	ns

The lack of any observable difference in the dose-response curves of the 5-HT syndrome following injection of 5-MeODMT at mid-light and mid-dark made the possibility of pharmacokinetic differences accounting for the circadian variation in head-twitch response unlikely. However, in an attempt to rule out this possibility more positively, the time course of both head-twitch and 5-HT syndrome response was studied at mid-light and mid-dark. At both these points of the light-dark cycle the number of head-twitches and the intensity of the syndrome were measured for alternate minutes for a period of 21 min. immediately following administration of 8 $mg.kg^{-1}$ 5-MeODMT. As can be seen in Figs. 20a and 20b the time of occurrence of the peaks measured at mid-light are not significantly different, but the number of head-twitches induced at mid-dark is greatly reduced, unlike the scores for the 5-HT syndrome. This further confirms the difference between these two behaviours over 24 hours.

The effect of the $5\text{-}HT_2$ antagonist pirenperone on head-twitches induced by 5-MeODMT was also studied at mid-light and mid-dark. Equipotent doses of 5-MeODMT at the two time points were chosen, and

Figure 20. Time-response curves for 5-MeODMT-induced head-twitches and syndrome at mid-light and mid-dark.



the antagonism of the head-twitch response to these doses by pirenperone was examined using the method described in Section 2.2.4.

Mice taken from the mid-light point in their light-dark cycle were given 16 $mg.kg^{-1}$ 5-MeODMT, while those from the mid-dark point were given 32 $mg.kg^{-1}$ 5-MeODMT. In vehicle pretreated mice these doses produced 17.2 $\pm$ 2.7 (mean $\pm$ s.e.m.) and 19.5 $\pm$ 1.5 head-twitches respectively. Pirenperone was found to have a very similar effect against these approximately equipotent doses as shown in Fig. 21. None of the differences reached statistical significance.

3.2.3 Effect of 5-HTP, quipazine and mescaline in mice

In order to ensure that it was not some property other than activity at the 5-HT_2 receptor that was responsible for the observed circadian variation in the 5-MeODMT induced head-twitch, the head-twitch induced by a series of 5-HT agonists was studied at the times of peak and trough activity. Dose-response curves to 5-HTP, quipazine and mescaline were constructed at mid-light and mid-dark. Thes dose-response curves were carried out as previously described in Section 2.2.

The results of these experiments are shown in Figs. 22 and 23. A shift to the right of the dose-response curves was observed when each of these drugs were tested at the mid-dark point compared to the mid-light point, although not all the differences were significant. As noted previously (Section 2.2.3) the maximum effect of quipazine and mescaline was substantially less than for 5-MeODMT or 5-HTP.

Figure 21. Inhibition of equipotent doses of 5-MeODMT at mid-light and mid-dark by pirenperone.

All values are mean ± s.e.m., n=8.

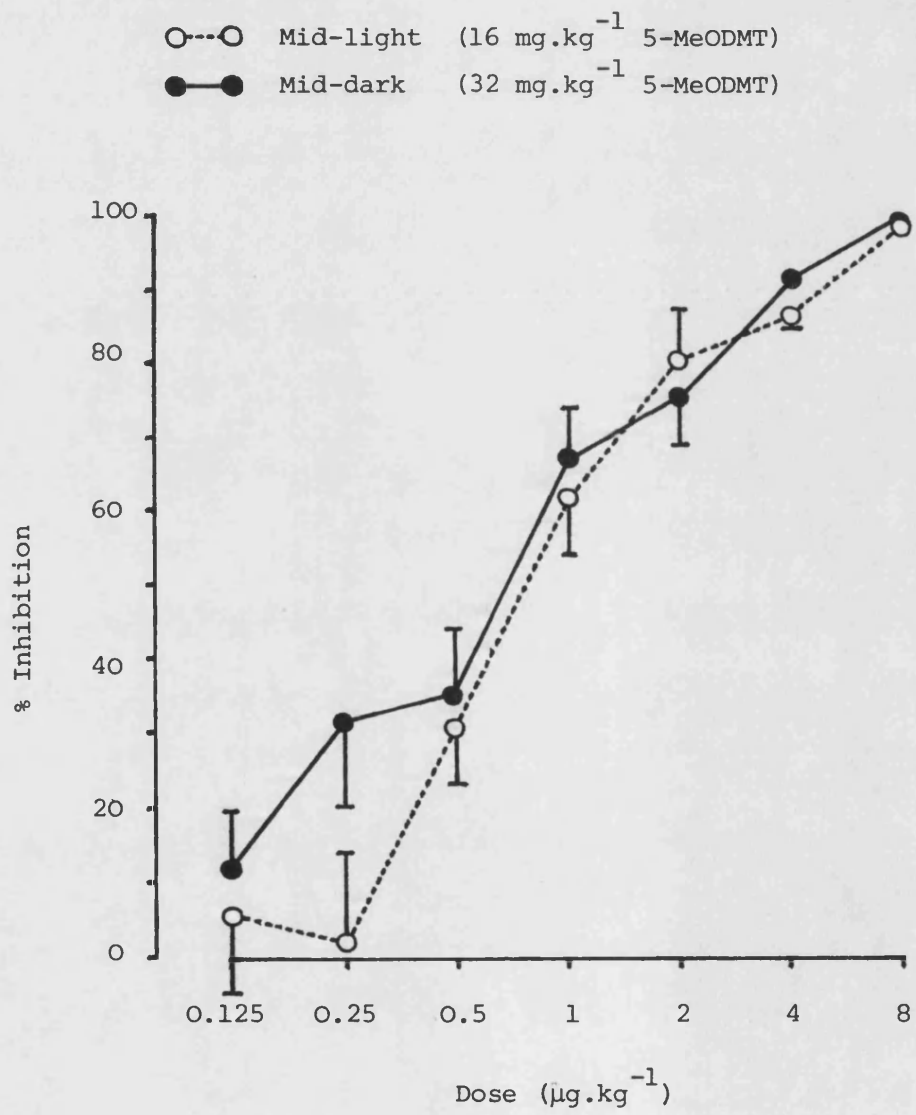


Figure 22. Dose-response curves for 5-HTP-induced head-twitch at mid-light and mid-dark in mice.

All values are mean ± s.e.m., n=7.

○—○ Mid-light ●—● Mid-dark

t-test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

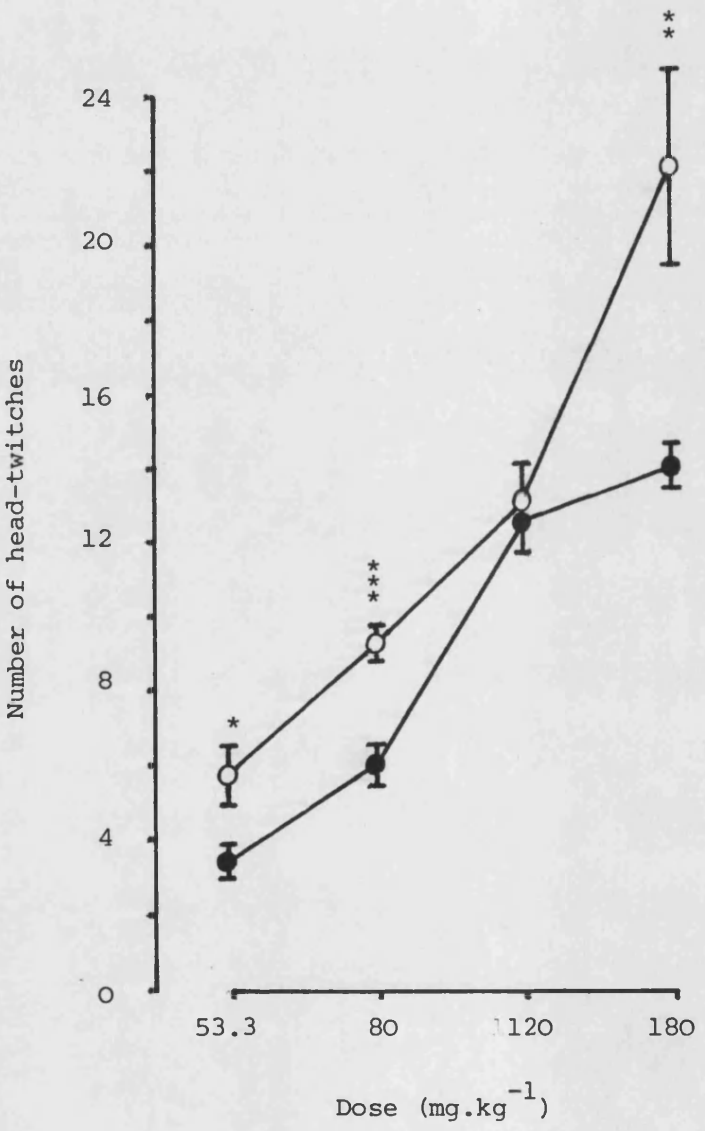
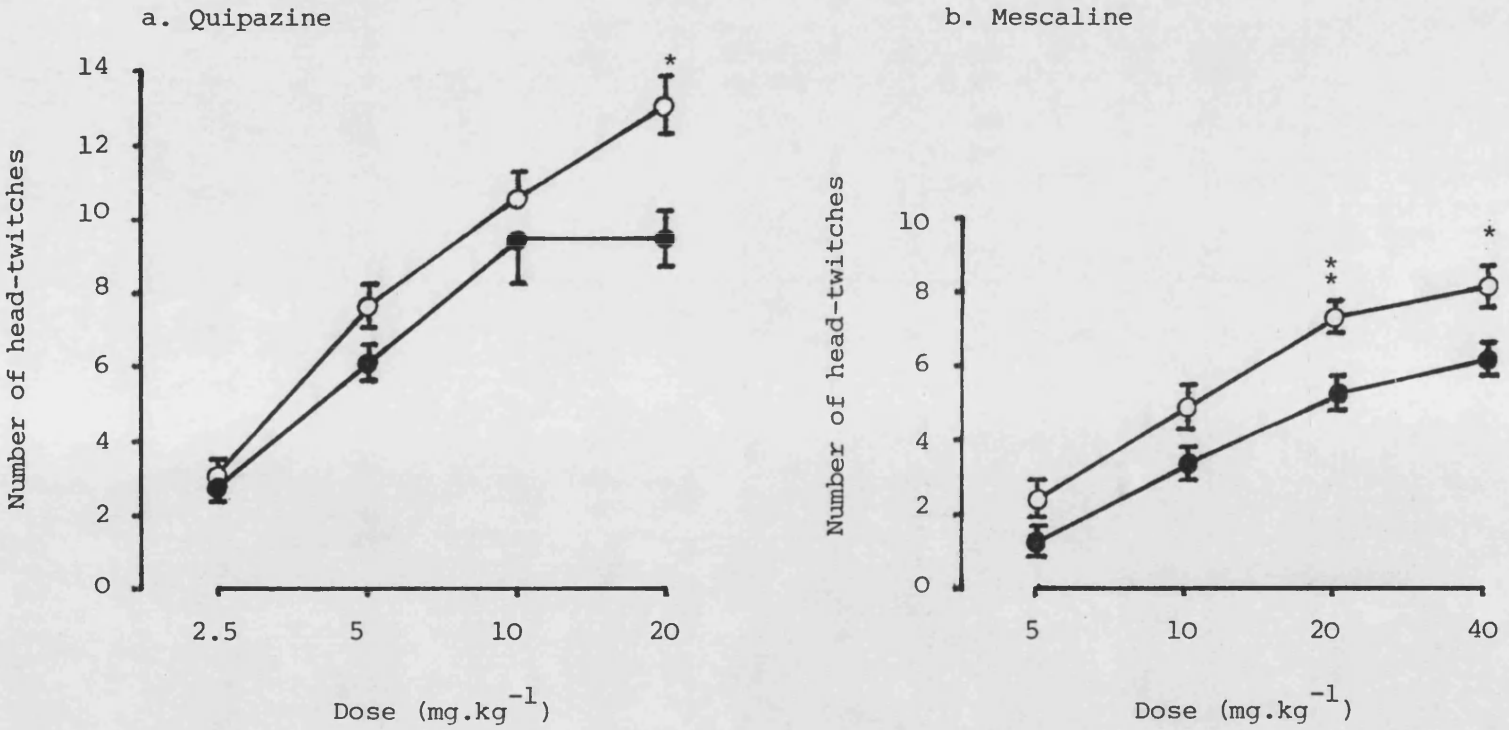


Figure 23. Dose-response curves for quipazine and mescaline-induced head-twitches at mid-light and mid-dark in mice.

All values are mean ± s.e.m., n=8. ○—○ Mid-light ●—● Mid-dark

t-test: * $p<0.05$, ** $p<0.01$



3.2.4 Effect of 5-HTP in the rat

In order to study the 24-hour variation in the head-twitch response to 5-HTP in the rat, different groups of 8 rats received 75 $mg.kg^{-1}$ 5-HTP following carbidopa as described in section 2.2.5. This dose of 5-HTP was approximately the ED_{50} in rats (Fig. 8a). The number of head-twitches made by each rat in a 10 min. period 30 min. after receiving 5-HTP was recorded.

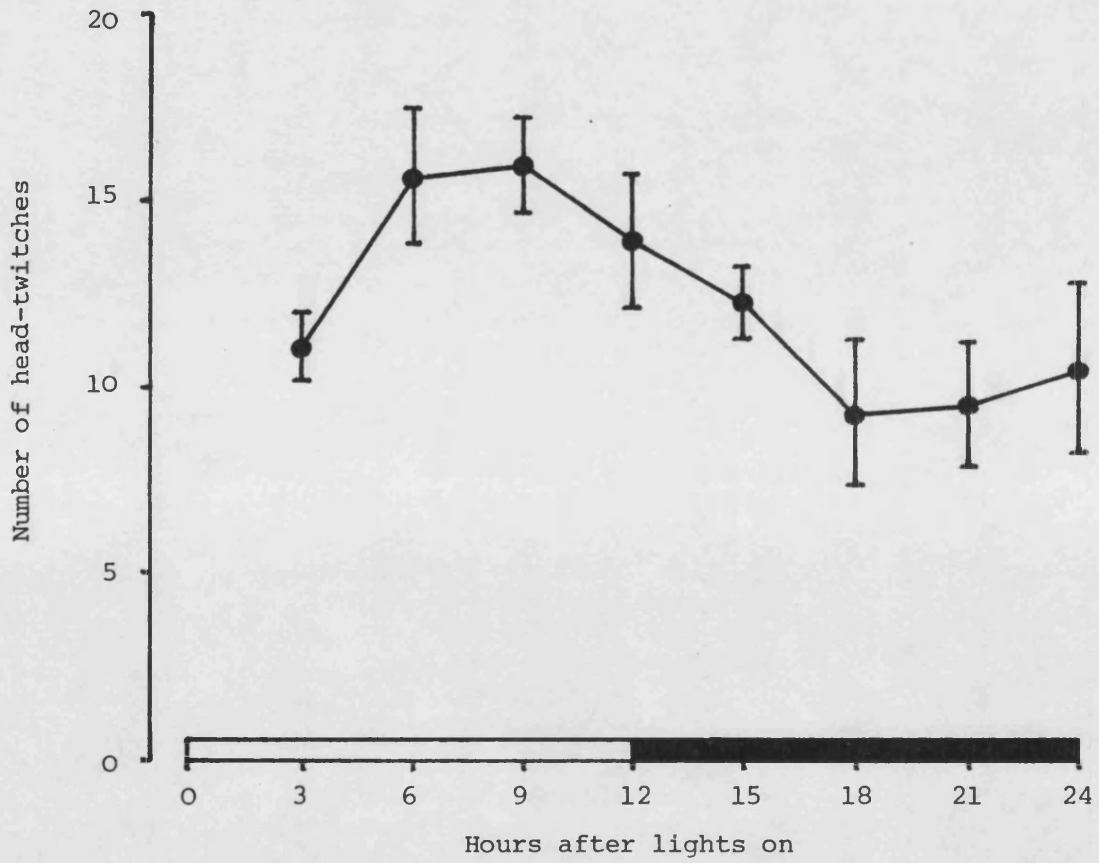
The head-twitch response was found to follow a clear 24-hour variation, with the times of peak and trough response occurring at similar times to those found for 5-MeODMT-induced head-twitches in the mouse (Fig. 24). Analysis of variance of the data showed that the means varied significantly over 24 hours ($F=2.48$ for 7,56 DF, $p<0.05$).

3.3 CIRCADIAN VARIATION IN THE DISCRIMINATIVE STIMULUS PROPERTIES OF 5-HTP

The results shown in Section 2.3 demonstrate that the discriminative stimulus properties of 5-HTP are dose-dependent and that changing the training dose has a direct effect on the time taken to learn the discrimination task. It therefore follows that if the sensitivity of the receptor mediating the stimulus changes, then an animals perception of that stimulus will change, altering the degree of stimulus control that the dose of training drug used will have. Thus by using the same training dose at different times during the 24 h cycle, any differences between the times taken to reach criterion for each group can be attributed to changes in sensitivity

Figure 24. 24-hour variation in the head-twitch response to 5-HTP in rats. (75 mg.kg^{-1})

All values are mean ± s.e.m., n=8. Black bar represents hours of darkness.



of the receptor and should allow the detection of a circadian variation in this parameter.

3.3.1 Methods

Using the training technique already described in Section 2.3.3, different groups of 8 rats were trained to discriminate 50 $mg.kg^{-1}$ 5-HTP from saline in the presence of 25 $mg.kg^{-1}$ carbidopa at 3 hourly intervals throughout the light-dark cycle. All training procedures were carried out under the prevailing lighting conditions of the designated clock hour, i.e. under normal room lighting during the light phase and under dim red light during the dark phase.

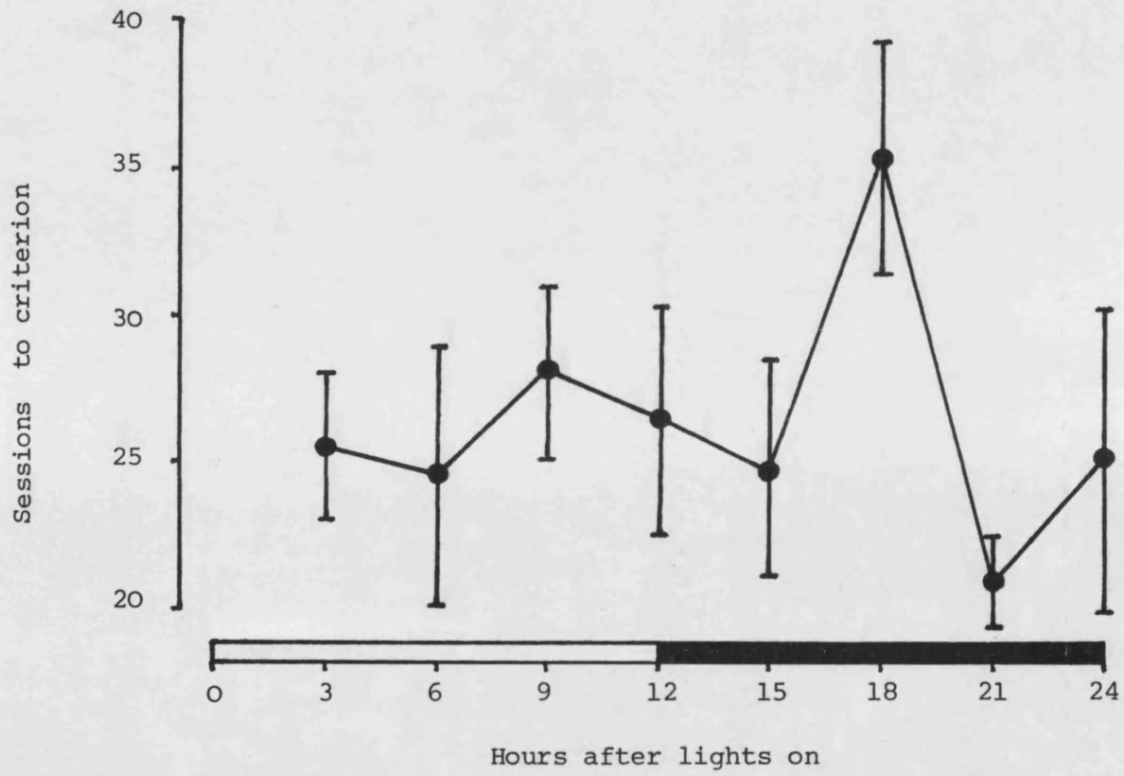
3.3.2 Results

Of the 64 rats tested, 4 failed to reach criterion within 50 sesions and so for the purposes of calculation these animals were assigned values of 50 sessions to criterion. Groups trained at +12 and +18 hours after lights on (HALO) contained one such animal each, and the group trained at +24 HALO contained two. It is difficult to know whether these animals failed to learn the discrimination task because of failure to detect the cue, or an inability to learn the task. Because of this their values were included in all calculations.

The variation in sessions to criterion over 24 hours is shown in Fig. 25 and no clear circadian rhythm is apparent. This was confirmed by analysis of variance of the data which indicated that none of the means differ significantly ($F=1.27$ for 7,56 DF, ns).

Figure 25. 24-hour variation in acquisition of the 5-HTP-saline discrimination in rats.

All values are mean ± s.e.m., n=8. Black bar represents hours of darkness.



3.4 CIRCADIAN VARIATION IN RU 24969 INDUCED HYPERACTIVITY

3.4.1 Methods

At 3 hourly intervals throughout the light-dark cycle, different groups of 8 mice received either 0.625 $mg.kg^{-1}$ RU 24969 or saline. The dose-response curve for RU 24969 (Fig. 15) shows that this dose produces an approximately half maximal increase in activity. Thirty minutes after RU 24969 administration the mice were tested by placing them, one at a time, in the centre of the open field, and the number of line crossings counted for the following 2 min period as previously described (Section 2.4.2).

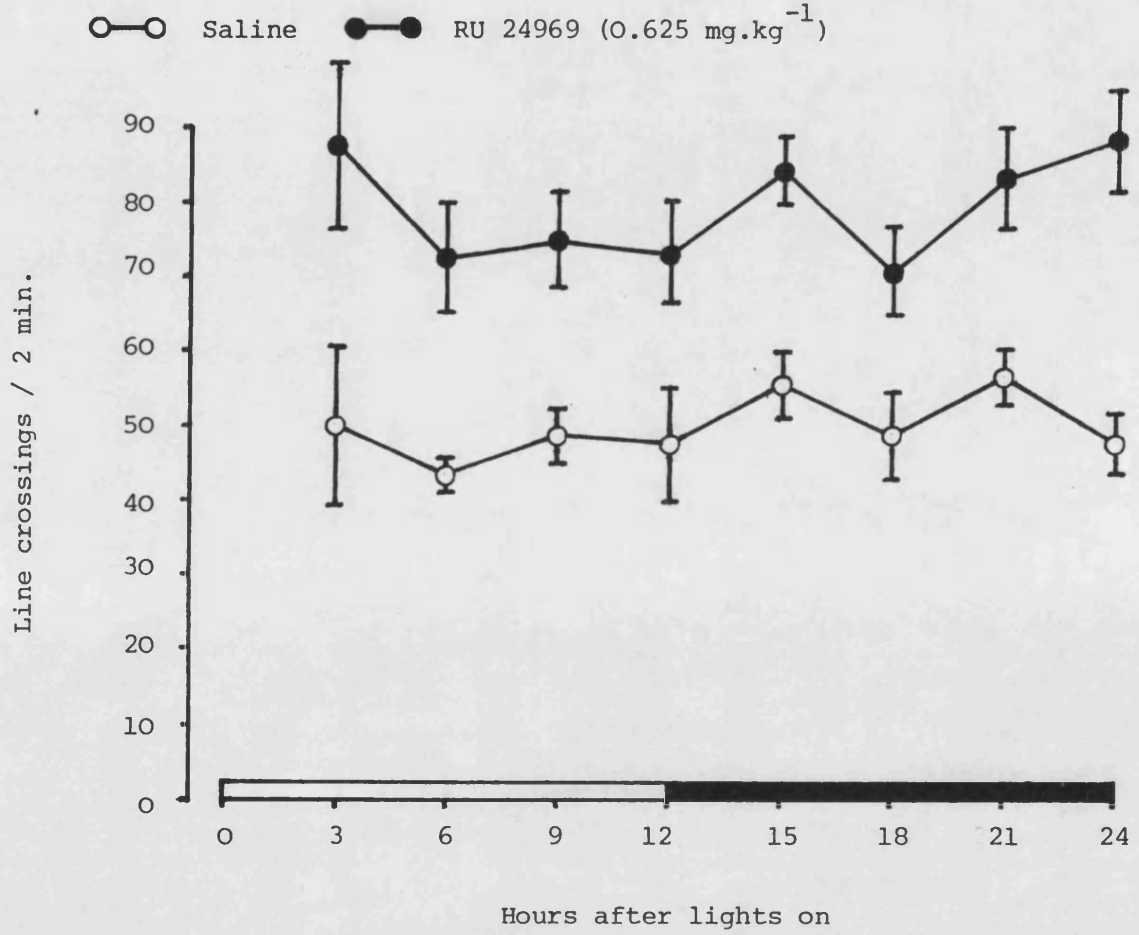
All measurements during the light phase were carried out under normal room lighting, while those during the dark phase were carried out under red light.

3.4.2 Results

The effect of RU 24969 on locomotor activity in the open field over 24 hours is shown in Fig. 26. The activity of saline treated mice was not found to have a noticable circadian rhythm in this procedure, and the degree of hyperactivity that RU 24969 induced similarly showed no change over 24 hours. This was confirmed by analysis of variance which showed that the amount of hyperactivity did not vary significantly ($F=0.98$ for 7,55 DF, ns).

Figure 26. 24-hour variation in RU 24969-induced hyperactivity in mice.

All values are mean ± s.e.m., n=8. Black bar represents hours of darkness.



3.5 CIRCADIAN VARIATION OF 8-OHDPAT INDUCED HYPOTHERMIA

3.5.1 Methods

At 3 hourly intervals throughout the light-dark cycle the rectal temperature of groups of 10 mice was measured as previously described (Section 2.5.2). The animals then received 0.5 $mg.kg^{-1}$ sc 8-OHDPAT, a dose which had previously been shown to induce approximately half the maximal hypothermia (Fig. 16). After 30 min the rectal temperature was again measured.

At two time points (+12 and +21 HALO) separate groups of mice were given saline vehicle sc. This was not found to produce a temperature change. All measurements were carried out under normal room lighting at an ambient temperature of 20-23^{o}C.

3.5.2 Results

The body temperature of untreated mice showed the expected circadian variation that has been previously described, with the peak occurring in the dark phase (e.g. Dunn et al., 1977) (Fig. 27). The same circadian rhythm of temperature was also apparent in the mice after 8-OHDPAT treatment but at approximately 1^{o}C lower at all times. The lower part of Fig. 27 shows how 8-OHDPAT affected body temperature over 24 hours; there is no sign of any rhythmic variation. This was confirmed by analysis of variance of the change in temperature ($F=0.464$ for 7,68 DF, ns).

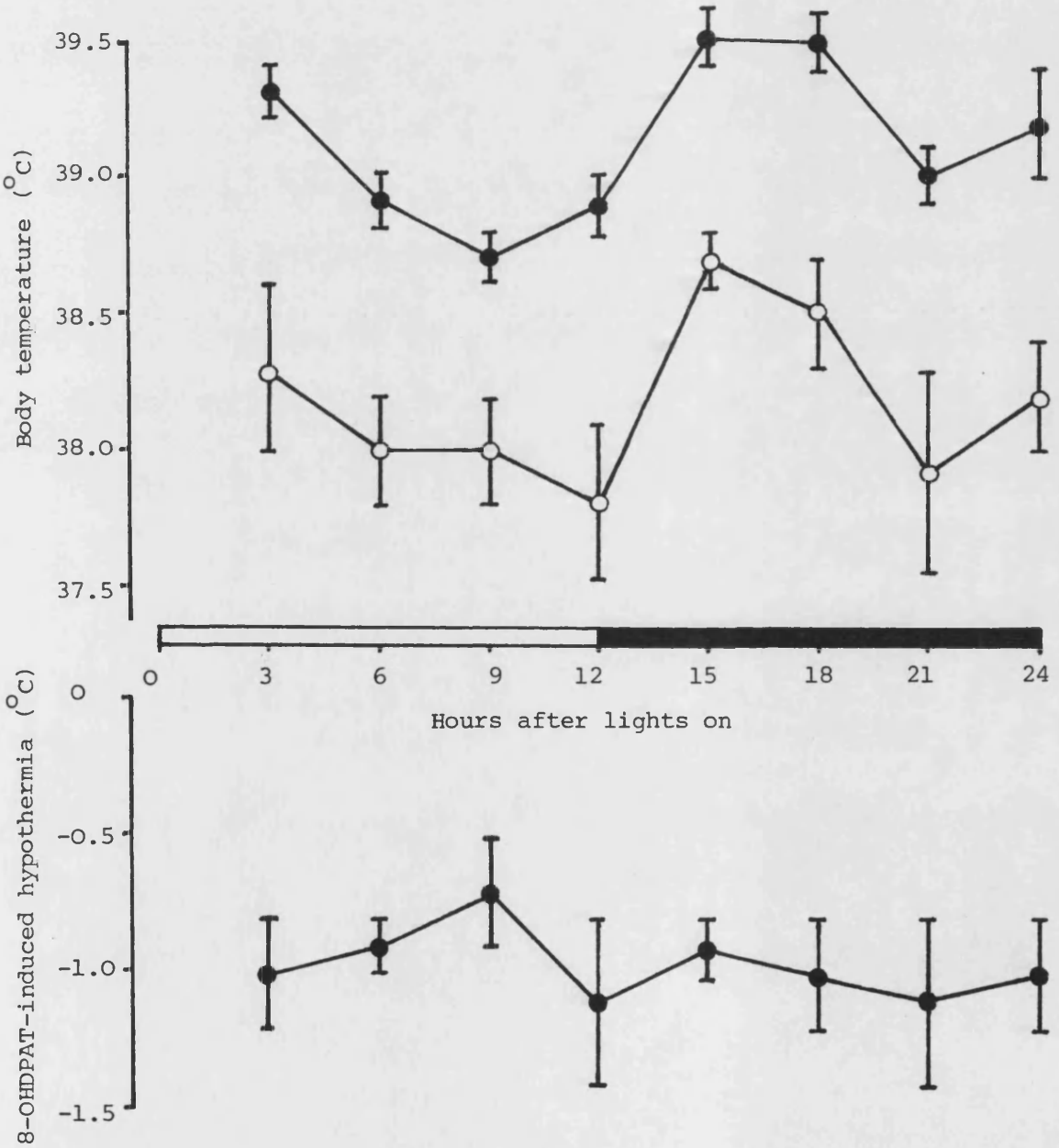
Figure 27. 24-hour variation in 8-OHDPAT-induced hypothermia in mice.

All values are mean ± s.e.m., n=10.

Black bar represents hours of darkness.

●—● Body temperature before 8-OHDPAT

○—○ Body temperature after 8-OHDPAT



3.6 DISCUSSION

The evidence that each of the behaviours examined over 24 hours in this chapter is mediated by a 5-HT receptor has already been discussed and will not be repeated here. Although there is still some doubt surrounding the classification of the receptors involved in some of them, there can be little doubt that some significant differences have emerged from these experiments between those behaviours thought to reflect activity at 5-HT_1 receptors and those reflecting activity at the 5-HT_2 receptor.

It is generally accepted that both the head-twitch response and the 5-HT syndrome elicited by injection of 5-MeODMT in mice result from direct stimulation of central 5-HT receptors (see section 1.4). The earlier experiments of Singleton and Marsden (1981) showed that the head-twitch response to this compound varied with time of testing. The results presented here clearly demonstrate that this is due to a well defined 24 hour rhythm in the head-twitch response, and also indicate that this rhythm does not result from a 24 hour variation in pharmacokinetic factors. There is a clear distinction, however, between the head-twitch and the 5-HT syndrome which did not show any signs of variation over 24 hours, either as total score, or when the individual components of the syndrome were examined.

The most notable difference between the head-twitch and the 5-HT syndrome is that the former is almost certainly mediated by the 5-HT_2 receptor subtype, whereas the latter is mediated by some other receptor, possibly that designated the 5-HT_1 (see section 1.4 and 2.6 for discussion). To ensure that it was the 5-HT_2 agonist properties of 5-MeODMT that were responsible for the observed

variation, a number of other 5-HT_2 agonists with varying properties at other sites were also examined. All of these were found to produce rightward shifts in their dose-response curves for induction of head-twitches when tested at mid-dark compared to mid-light. Another feature of all these dose-response curves was the reduced maximum effect at mid-dark. This may be indicative of a decrease in receptor number rather than affinity, a conclusion born out by the effect of pirenperone against equipotent doses of 5-MeODMT at mid-light and mid-dark. At both times, the effectiveness of pirenperone was the same. Examination of the head-twitch response to 5-HTP in the rat confirmed the 24 hour variation observed in mice, with both rhythms exhibiting a peak around mid-light and a trough around mid-dark.

Further examination of the 5-HT_1 receptor over 24 hours confirmed the results obtained with the syndrome. Neither 8-OHDPAT-induced hypothermia, RU 24969-induced hyperactivity, nor the 5-HTP discriminative stimulus showed any evidence of a significant 24 hour variation. It is unlikely that all these behaviours are mediated by the same receptor but the most obvious conclusion from the results presented in this chapter is that behaviours mediated by the 5-HT_2 receptor display a circadian periodicity, while those that are mediated by other 5-HT receptor subtypes do not.

The simplest explanation for the 24 hour variation in the head-twitch response is an underlying rhythm in 5-HT_2 receptor function. This is supported by the results of Bruinink et al. (1983) who have demonstrated a marked circadian variation of 5-HT_2 binding sites in rat forebrain using [^{3}H]-spiperone as the ligand. This variation, like that of the head-twitch response in both rats and mice had a

maximum in the light phase and a minimum in the dark phase. It seems reasonable to suppose that a similar variation in the 5-HT_2 receptors responsible for initiation of the head-twitch response is responsible for the results presented here. However, as already pointed out, the head-twitch response appears to be mediated by hindbrain mechanisms, and variations in the numbers of 5-HT_2 binding sites in this brain area have not been studied. The variation in the numbers of head-twitches would suggest that they behave in a manner similar to the 5-HT_2 sites examined in the forebrain by Bruinink et al. (1983).

The binding of [^{3}H]-5-HT to membrane fractions from whole rat brain has also been studied over 24 hours and the authors of this report claim that a circadian rhythm is apparent (Wesemann et al., 1983). If this is so it would imply that the circadian variation in behaviours mediated by these receptors is damped out at some point. It is known that other neurotransmitter systems can modulate all the behaviours examined in this chapter, and this may be responsible for the lack of 24 hour variation in some of them, although there is no direct evidence for this.

However, the conclusions of Wesemann et al., (1983) cannot be taken at face value, as the circadian rhythm that they observe is not very marked, although significant differences are present. What is perhaps more important is the demonstration that [^{3}H]-5-HT binds to more than one subtype of the 5-HT receptor (section 1.3) and no information is available on how these may interact to produce the variations observed by Wesemann et al., (1983). Until more detailed information on the variation of binding to specific subtypes of the 5-HT_1 receptor over 24 hours is available their results should

be viewed with caution. It should be noted here that all receptor types so far studied using ligand binding techniques over 24 hours have been shown to undergo circadian variation (Kafka et al., 1981, 1983).

The simplest conclusion from the results presented in this chapter is that 5-HT_2 but not 5-HT_1 receptors show a circadian variation, either in number or activity. The remainder of this discussion will concentrate on the evidence and possible mechanisms for this.

The first problem that needs to be addressed is that of the mechanisms involved in the variation of 5-HT_2 receptor activity. It is clear that the rhythm of head-twitch activity closely parallels the daily variations in 5-HT levels. It was speculated in section 1.2 that one of the primary reasons for this was a marked variation in 5-HT utilisation. This is significantly higher during the dark-period than during the light-period. Therefore, when 5-HT release, and presumably 5-HT neuronal activity, is highest, the receptors are least sensitive and vice versa. This arrangement would mean that cells post-synaptically located to 5-HT neurones could detect changes in neuronal activity more readily. If the post-synaptic receptors were most sensitive when neuronal activity was highest, and least sensitive when it was lowest, it could result in the post-synaptic cell being maximally stimulated during the dark-phase and being unresponsive during the light-phase. In this condition any changes in presynaptic activity are likely to go undetected.

It is therefore likely that the changes in receptor number are driven by presynaptic changes. Thus the 5-HT_2 receptors display

rapid 'down-regulation' when presynaptic activity is highest and 'up-regulation' when it is lowest. The ontological data would support this conclusion. The rhythm of 5-HT concentrations is not fully developed until the 35th day of life in rats (Okada, 1971), whereas the rhythm in 5-HT_2 binding sites is not apparent at 30 days but is fully developed by the 90th day (Bruinink et al., 1983). Unfortunately it is not known how the rhythm in 5-HT release develops.

Clearly the mechanism for this rapid adaptation response will be different to that frequently reported following administration of agonists or antagonists for a prolonged period of time. In general this process requires a period of days, and reversal probably requires the synthesis of new receptors. The half-life for recovery of the α_1-receptor after phenoxybenzamine treatment for example is around 1.9 days, which is far slower than the changes of a similar magnitude seen over 24 hours (McKernan and Campbell, 1982).

Possible mechanisms for the rapid changes in receptor number over 24 hours have been discussed by Campbell et al. (1985). It has been shown that β-receptors can move in and out of the surface membrane of cells as a result of the formation of small vesicles by invagination of the cell membrane (Harden, 1983). This process may be the forerunner of the longer term desensitisation, but as it is a rapid process it could certainly be the mechanism for the rapid circadian changes that are observed. Rapid changes in β-adrenoceptor responses have been reported by Lefkowitz et al. (1976) and a similar mechanism may be involved in the changes seen in the case of 5-HT_2 receptors. In support of this it has recently been demonstrated that 5-HT_2 receptors can display a rapid down-

regulation, within 3 hours of administration of the 5-HT-uptake inhibitor paroxetine (Koshikawa et al., 1985).

If these rapid changes are related to, or are forerunners of, the longer term desensitisation of receptor activity that have been reported then one would expect a difference in regulatory ability between 5-HT_1 and 5-HT_2 receptors when studied over longer periods. Rapid changes of the 5-HT_1 site have not been studied, but there are a number of reports which indicate that it is much easier to change 5-HT_2 receptor activity than 5-HT_1 receptor activity.

Chronic treatment with LSD, which binds to both 5-HT_1 and 5-HT_2 receptors, was found to decrease its binding to 5-HT_2 receptors in all brain regions studied, with the largest changes occurring in the diencephalon midbrain area. However the binding of LSD to 5-HT_1 sites was unaffected in all the brain regions examined (Buckholtz et al., 1985). Treatment with a variety of antagonists paradoxically reduced the number of 5-HT_2 binding sites but left the number of 5-HT_1 sites unaltered (Blackshear et al., 1983). In 1978, Wirz-Justice et al. failed to modify serotonin receptor sensitivity, as measured by [^{3}H]-5-HT binding, by either chronic 5-HTP, chronic clomipramine or chronic metergoline treatment. Dorsal raphe lesions with 5,7-DHT also failed to affect 5-HT binding in the cortex (Whittaker and Deakin, 1981), but have been reported to increase the concentration of 5-HT binding sites in the substantia nigra (Blackburn et al., 1984). Treatment with fenfluramine for 14 days has also been shown to affect [^{3}H]-5-HT binding sites (Samanin et al., 1980). Overall, these results point to a greater difficulty in changing the sensitivity of 5-HT_1 receptors compared to 5-HT_2 receptors. Alternatively, there may be regional differences which

have not been fully explored.

The differences between 5-HT sites with regard to up- or down-regulation are also apparent from behavioural studies. Nisbet and Marsden (1984) have recently reported that lesions of brain 5-HT with intracerebral 5,7-DHT enhance the behavioural response to 5-MeODMT, but not the hyperactivity following RU 24969. The 5-MeODMT behaviour that was measured was the 5-HT syndrome, which results presented in this thesis (section 2.2) suggest is mediated by a 5-HT_1 receptor subtype. However, other workers have suggested that when the syndrome is induced by 5-MeODMT it is mediated by the 5-HT_2 receptor as it can be inhibited by 5-HT_2 antagonists (Green and Heal, 1985). These differences in results may be due to strain differences, which have been reported in rats (Jones and Dourish, 1982).

While no clear concensus is reached by analysis of these reports they clearly suggest that there are differences in the regulatory ability of different 5-HT receptors. If 5-HT release shows a similar rhythm in all brain areas, and there is no reason at present to suppose that it does not, then from the results presented in this chapter one would predict that behaviours depending on activation of 5-HT_1 receptors for their expression would be more likely to display a circadian rhythm than those that employ the 5-HT_2 receptor. This is because in 5-HT_1 receptor mediated effects the presynaptic rhythmicity will not be damped out by compensatory changes. Thus the observed rhythm in head-twitch activity may be an artefact of an adaptive response to presynaptic activity.

There are a number of assumptions built into the scenario just described, and clearly a lot more work is needed to elucidate the

reasons for the observed variation in head-twitch response but not in other 5-HT receptor mediated behaviours.

4 BENZODIAZEPINES AND 5-HT

4.1 INTRODUCTION

The interaction of benzodiazepines (BDZs) with the brain 5-HT system has already been discussed in section 1.5, and the evidence points to an action of BDZs at two sites. The experiments described in this chapter were designed to elucidate the role of these pre- and postsynaptic actions of BDZs in their effects on the head-twitch response. To study the interaction of BDZs with postsynaptic 5-HT mechanisms, their effect on the response to the direct 5-HT receptor agonist 5-MeODMT was studied, and to investigate the presynaptic effects of BDZs, the indirect agonist 5-HTP was used. This latter agent is the natural precursor of 5-HT and requires presynaptic decarboxylation before it can act to induce head-twitches. The properties of these two 5-HT agonists are discussed in more detail in chapter 2.

Four BDZs were chosen for this study to provide a variety of structures and activities. Diazepam was chosen as it is one of the most commonly used standard BDZs and there is a large quantity of data available on it. In the clinic it is used as an anxiolytic, anti-epileptic and muscle relaxant. Clonazepam is one of the BDZs used by Nakamura and Fukushima (1976) that induced head-twitches when given alone. Its clinical use is restricted to treating epileptic states. Oxazepam is an N-demethylated 3-hydroxylated metabolite of diazepam. The polar group at the 3-position means it has a much shorter duration of action and because of this it is used for the treatment of acute panic attacks. Finally, the 1,5-BDZ clobazam was included in the group. This drug has a similar clinical profile to oxazepam, but has a longer duration of action and can be used in the treatment of chronic anxiety.

All information regarding the clinical use of the BDZs was obtained from the British National Formulary (1984).

Also used in this study were agents that act upon the GABAergic system. These include the $GABA_A$ antagonist bicuculline, the $GABA_A$ agonist muscimol and the GABA-transaminase inhibitor amino-oxyacetic acid. Also tested were the BDZ antagonists Ro 15-1788 and β-CCE.

4.2 MATERIALS

4.2.1 Animals

In all experiments reported in this chapter, male CFLP mice (25-40g) were used. They were housed in the stock rooms of the University of Bath animal house on 14h light-10h dark cycle, with lights on at 0500 hours. For the study of 24-hour variation the mice were housed as described in chapter 3. Food and water were freely available at all times.

4.2.2 Drugs

Details of dose volume, routes of administration and vehicles used for many of the drugs have already been given in section 2.1.

The BDZs and BDZ antagonists were suspended in 0.2% tragacanth solution. All other compounds were disolved or suspended in 0.9% saline.

The drugs used in these experiments, in addition to those already mentioned, with abbreviations if used, and source in brackets were: diazepam (Hoffmann La Roche); clonazepam (Hoffmann La Roche); oxazepam (Wyeth Laboratories); clobazam (Hoechst); ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo(1,5-a)(1,4) benzo-

diazepine-3-carboxylate (Ro 15-1788, Hoffmann La Roche); β-carboline-3-carboxylate ethyl ester (β-CCE, Research Biochemicals Inc.); muscimol (Sigma); amino oxyacetic acid (AOAA, Sigma). (+)-bicuculline (Sigma); para-chlorophenylalanine HBr (pCPA, Sigma); diaminobutyric acid (DABA, Sigma).

4.3 EFFECT OF BENZODIAZEPINES ON 5-MeODMT-INDUCED BEHAVIOURS

4.3.1 Methods

The method used to evaluate 5-HT mediated behaviours induced by 5-MeODMT were exactly as previously described in section 2.2. A dose of 2.5 $mg.kg^{-1}$ 5-MeODMT was used throughout except where stated. The pretreatment time before the observation period for each compound used in the following experiments was as follows:
All BDZs: 60 mins; Ro 15-1788: 20 mins; β-CCE: 30 mins; bicuculline: 30 mins.

4.3.2 Effect of benzodiazepines on 5-MeODMT-induced head-twitches

Following administration of the doses of BDZs used in this study the mice appeared very sedated and in addition had decreased muscle tone. It was anticipated that these effects would interfere with any direct interaction between BDZs and the induction if head-twitches, but this did not appear to be the case except at the highest doses used.

The effect of the four BDZs on head-twitches induced by 2.5 $mg.kg^{-1}$ 5-MeODMT is shown in Fig. 28; a significant dose-related potentiation of this behaviour is apparent, with a tailing off of the response at the highest doses. Diazepam, oxazepam and clonazepam were approximately equipotent in this experiment while clobazam was slightly more potent, all produced a similar maximum potentiation of head-twitches.

In the same experiment the effect of BDZs on the 5-HT syndrome induced by 5-MeODMT was also evaluated. None of the BDZs tested had a noticable effect on this parameter (Table 6). In the light of this result, the 5-HT syndrome was not rated in subsequent experiments.

Figure 28. Potentiation of 5-MeODMT-induced head-twitches by benzodiazepines.

All values are mean ± s.e.m., n=8.

t-test (v. vehicle): * p<0.05, ** p<0.01, *** p<0.001

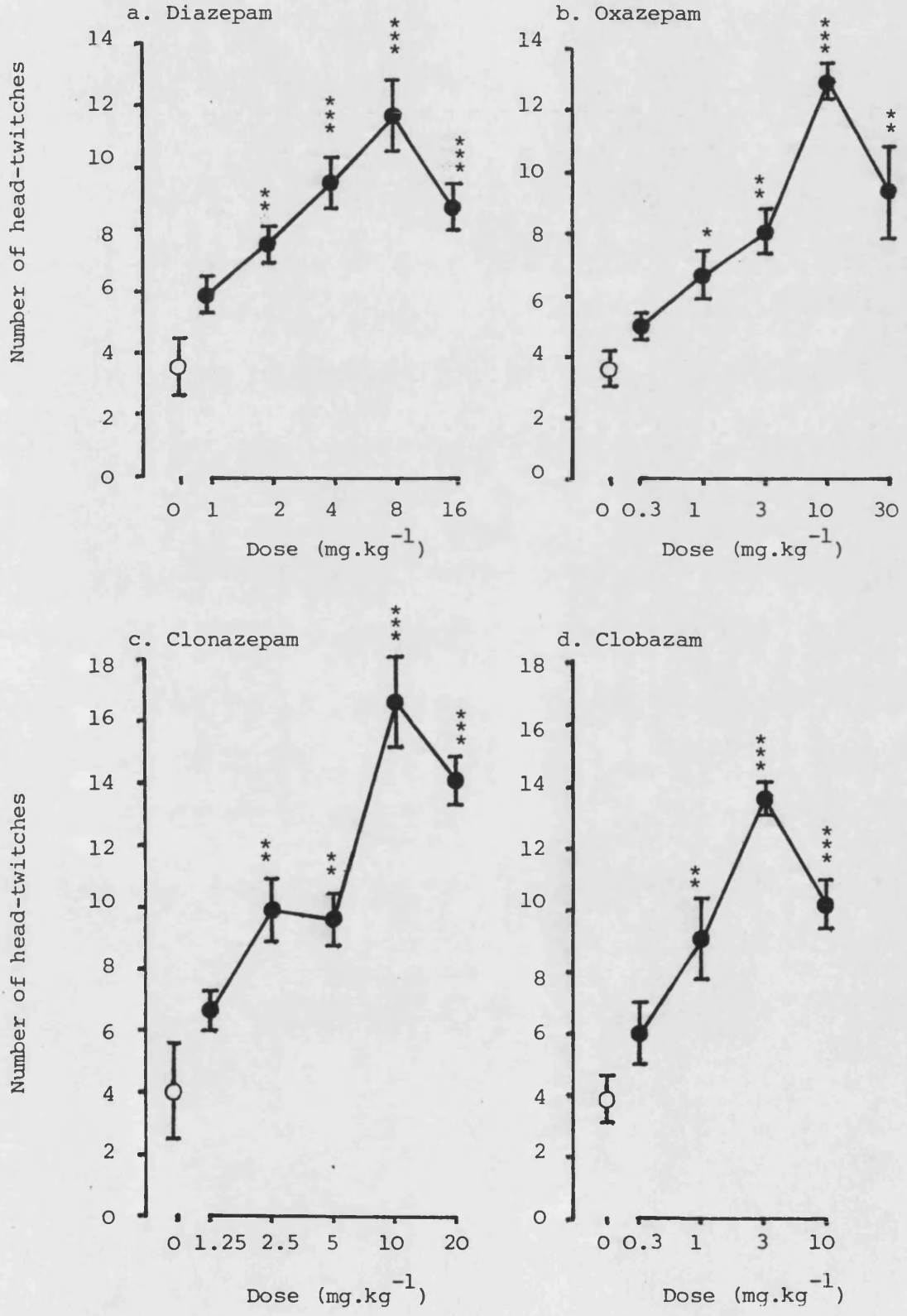


Table 6 Effect of benzodiazepines on the 5-MeODMT-induced 5-HT syndrome.

Treatment	Dose ($mg.kg^{-1}$)	Median score	Range
Vehicle	-	4	2-6
Diazepam	2	5	3-5
	4	4.5	3-8
	8	6	4-7
	16	5	4-7
Vehicle	-	4.5	2-6
Oxazepam	1	5.5	3-8
	3	6.5	5-8
	10	5	3-8
	30	4.5	3-6
Vehicle	-	5.5	5-9
Clonazepam	2.5	6	4-7
	5	6	5-8
	10	6.5	6-8
	20	7	5-8
Vehicle	-	6	4-7
Clobazam	0.3	5	3-6
	1	5.5	4-7
	3	6	4-8
	10	6.5	4-8

To ensure that the BDZs were not inducing head-twitches themselves, the doses that produced the maximum potentiation of 5-MeODMT head-twitches were tested for their ability to induce head-twitches. As can be seen from Table 7 only clonazepam induced a significant number of head-twitches, but this was not enough to account for the potentiation of 5-MeODMT-induced head-twitches.

Table 7 Induction of head-twitches by benzodiazepines

Treatment	Dose ($mg.kg^{-1}$)	n	Head-twitches (mean ± s.e.m.)	t-test
Vehicle	-	12	0.25 ± 0.13	
Diazepam	8	12	0.33 ± 0.19	ns
Clonazepam	10	12	1.20 ± 0.35	$p<0.05$
Clobazam	3	12	0.25 ± 0.13	ns
Oxazepam	10	12	0.10 ± 0.10	ns

4.3.3 Antagonism of benzodiazepine effect

In order to investigate the potentiation of 5-MeODMT-induced head-twitches by BDZs, the effect of clonazepam at 10 $mg.kg^{-1}$ was studied in more detail as this treatment produced the greatest effect.

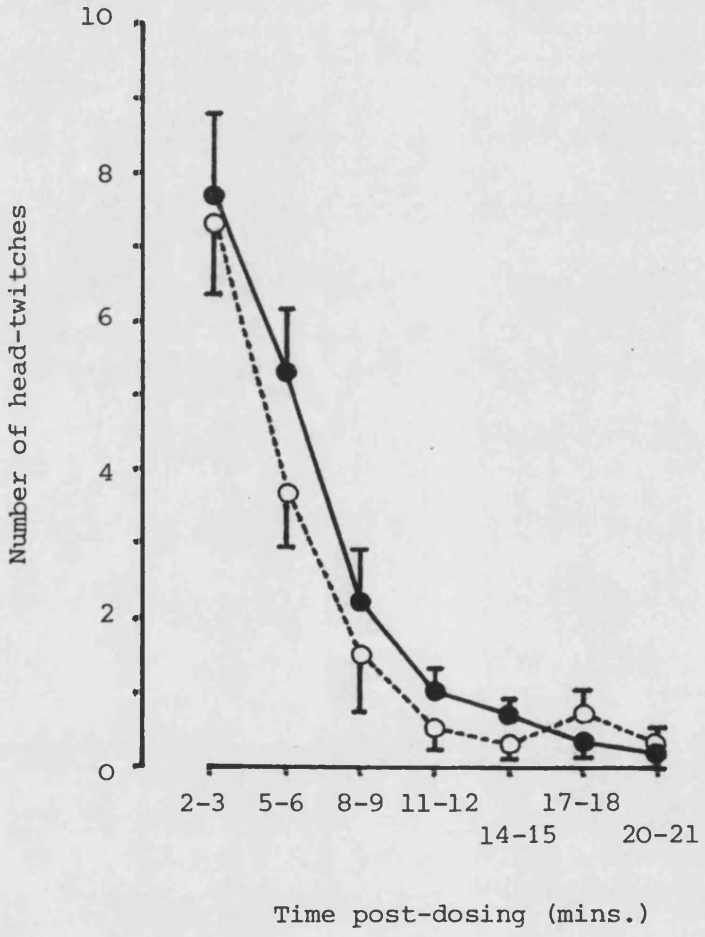
Pretreatment with clonazepam did not affect the time course of 5-MeODMT-induced head-twitches (Fig. 29). When 10 $mg.kg^{-1}$ in combination with 2.5 $mg.kg^{-1}$ 5-MeODMT was compared with 16 $mg.kg^{-1}$ 5-MeODMT the time response curves were not significantly different,

Figure 29. Effect of clonazepam on the time-course of 5-MeODMT-induced head-twitches.

All values are mean ± s.e.m., n=6.

○—○ 5-MeODMT (16 mg.kg^{-1})

●—● 5-MeODMT (2.5 mg.kg^{-1}) + Clonazepam (10 mg.kg^{-1})



indicating that the presence of the BDZ did not affect the pharmacokinetics of 5-MeODMT. This is important, as BDZs are known to prevent the exit of 5-hydroxyindole acetic acid from the brain, for example (Chase et al., 1970).

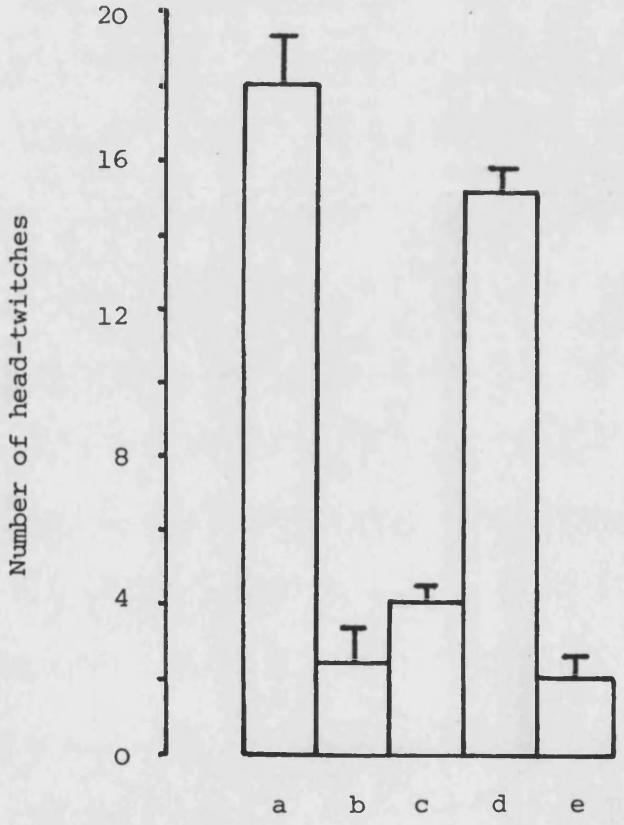
A variety of treatments were tested against the combination of clonazepam (10 $mg.kg^{-1}$) and 5-MeODMT (2.5 $mg.kg^{-1}$) and the results from these experiments are shown in Figs. 30 to 32. The data in Fig. 30 shows the effect of pirenperone. This 5-HT_2 antagonist is a potent inhibitor of the head-twitch response, as the effect of 5 $\mu g.kg^{-1}$ against 16 $mg.kg^{-1}$ 5-MeODMT shows. The same dose of pirenperone has a similar effect against the combination of clonazepam and 5-MeODMT.

Pretreatment with the tryptophan hydroxylase inhibitor, pCPA, did not inhibit the potentiation of head-twitches by clonazepam (Fig. 31). Mice received 300 $mg.kg^{-1}$ pCPA 48, 24 and 2 hours before the observation period, a pretreatment schedule which has been shown to reduce brain 5-HT by at least 45% in mice (Pratt et al., 1985). A slight potentiation of head-twitches was observed after pCPA, possibly as a result of postsynaptic 5-HT receptor supersensitivity following depletion of 5-HT.

The effect of bicuculline pretreatment on the clonazepam potentiation of 5-MeODMT-induced head-twitches is shown in Fig. 32a. Doses of 2 or 4 $mg.kg^{-1}$ bicuculline were not found to significantly affect the number of head-twitches observed. A similar lack of effect was also observed with the BDZ antagonists Ro 15-1788 (100 $mg.kg^{-1}$) and β-CCE (100 $mg.kg^{-1}$) as shown in Figs. 32b and 32c. Those animals treated with Ro 15-1788 or β-CCE appeared to be considerably less sedated than vehicle-pretreated mice.

Figure 30. Inhibition of clonazepam-potentiated 5-MeODMT-induced head-twitches by pirenperone.

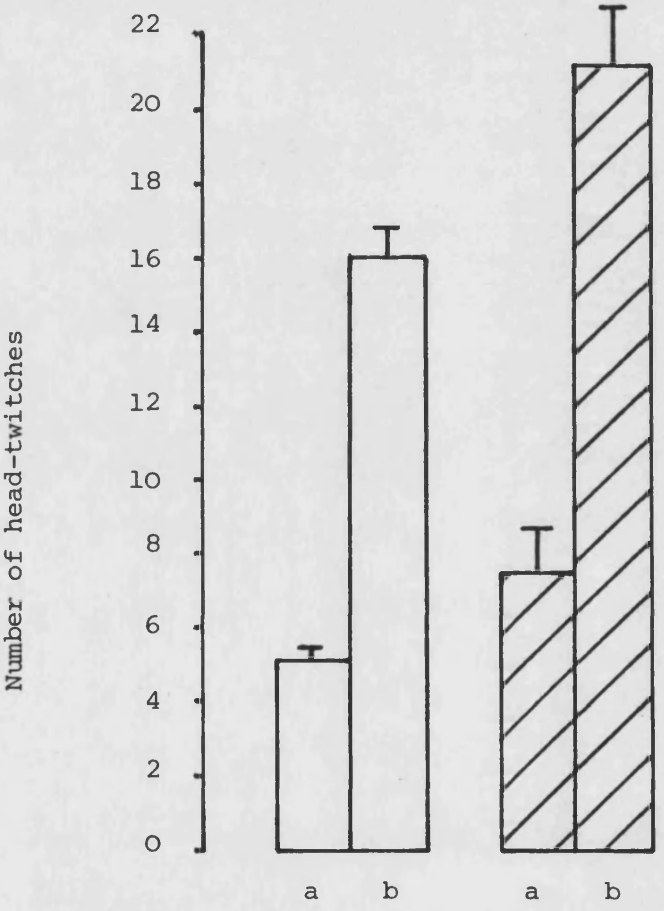
All values are mean ± s.e.m., n=8.



a. 5-MeODMT (16 $mg.kg^{-1}$)
b. 5-MeODMT (16 $mg.kg^{-1}$) + Pirenperone (5$\mu g.kg^{-1}$)
c. 5-MeODMT (2.5 $mg.kg^{-1}$)
d. 5-MeODMT (2.5 $mg.kg^{-1}$) + Clonazepam (10 $mg.kg^{-1}$)
e. 5-MeODMT (2.5 $mg.kg^{-1}$) + Clonazepam (10 $mg.kg^{-1}$)
+ Pirenperone (5$\mu g.kg^{-1}$)

Figure 31. Effect of pCPA pretreatment on the clonazepam potentiation of 5-MeODMT-induced head-twitches.

All values are mean ± s.e.m., n=10.



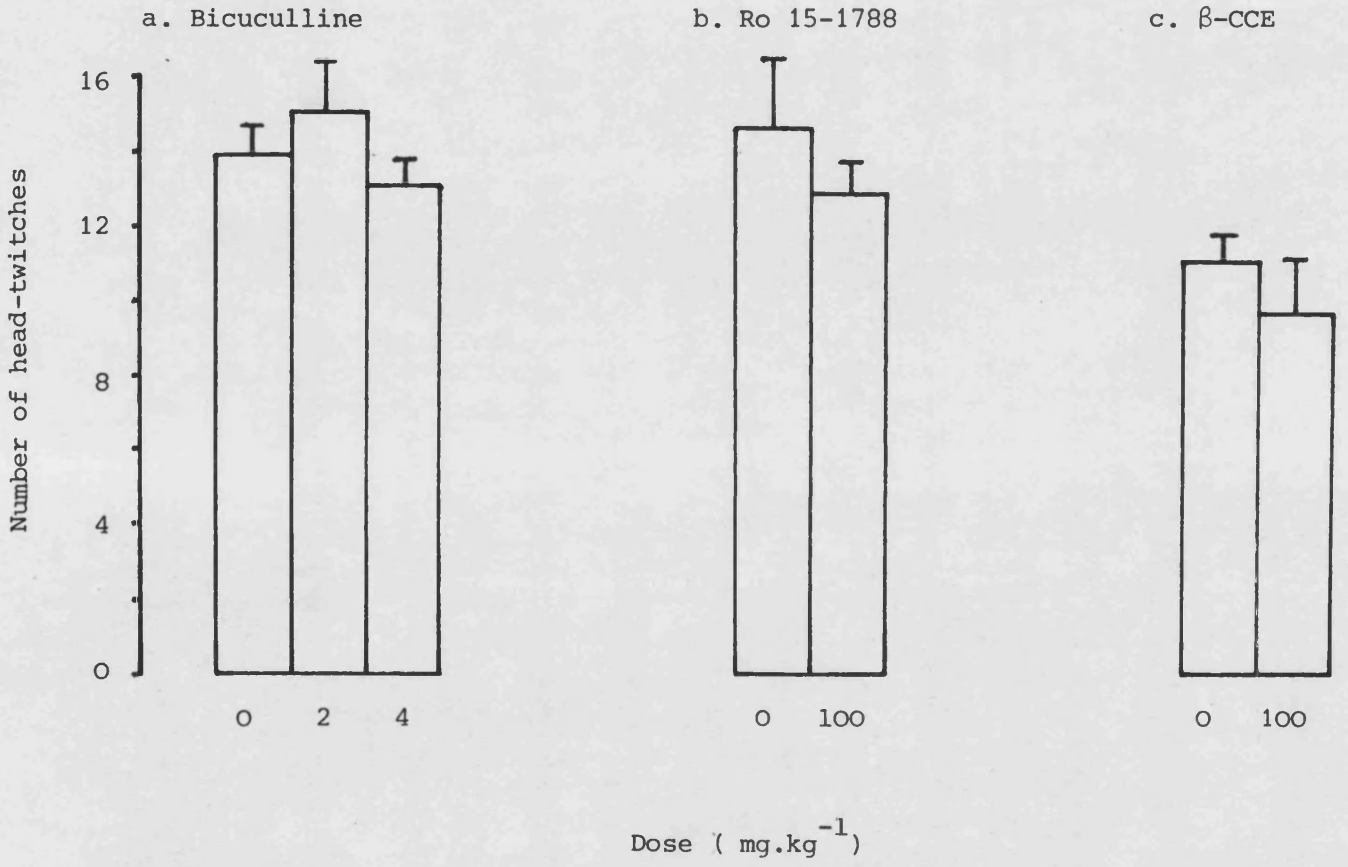
a. 5-MeODMT (2.5 $mg.kg^{-1}$)

b. 5-MeODMT (2.5 $mg.kg^{-1}$) + Clonazepam (10 $mg.kg^{-1}$)

pCPA pretreatment (3 x 300 $mg.kg^{-1}$)

Figure 32. Effect of bicuculline, Ro 15-1788 and β-CCE on clonazepam-potentiated 5-MeODMT-induced head-twitches.

All values are mean ± s.e.m., n=8.



4.4 BENZODIAZEPINES AND 5-HTP-INDUCED HEAD-TWITCHES

4.4.1 Methods

Head-twitches were induced in mice with a combination of carbidopa (25 $mg.kg^{-1}$) and 5-HTP as previously described in section 2.2. Doses of 50 $mg.kg^{-1}$ and 200 $mg.kg^{-1}$ 5-HTP were used for studying potentiation and inhibition of head-twitches respectively.

All abbreviations and pretreatment times were as previously described.

4.4.2 Effect of benzodiazepines on the 5-HTP-induced head-twitch

The effect of the four BDZs used in this study on head-twitches induced by 50 $mg.kg^{-1}$ 5-HTP is shown in Fig. 33. In contrast to their potentiation of 5-MeODMT-induced head-twitches, there was no significant potentiation of 5-HTP-induced head-twitches by any of the BDZs. The only significant effect observed was in fact an inhibition of head-twitches by clonazepam.

Following the demonstration of a lack of potentiation of 5-HTP-induced head-twitches by the BDZs, they were tested against 200 $mg.kg^{-1}$ 5-HTP in order to evaluate the significance of the inhibition observed against 50 $mg.kg^{-1}$ 5-HTP. The results for diazepam are shown in Fig. 34, and those for the other BDZs are shown in Table 8. Against the higher dose of 5-HTP only diazepam was found to inhibit the head-twitches, and this only reached 50% inhibition at the highest dose of 30 $mg.kg^{-1}$.

Figure 33. Effect of benzodiazepines on head-twitches induced by 5-HTP (50 mg.kg^{-1}).

All values are mean ± s.e.m., n=8.

t-test (v. vehicle): * $p<0.05$, ** $p<0.01$

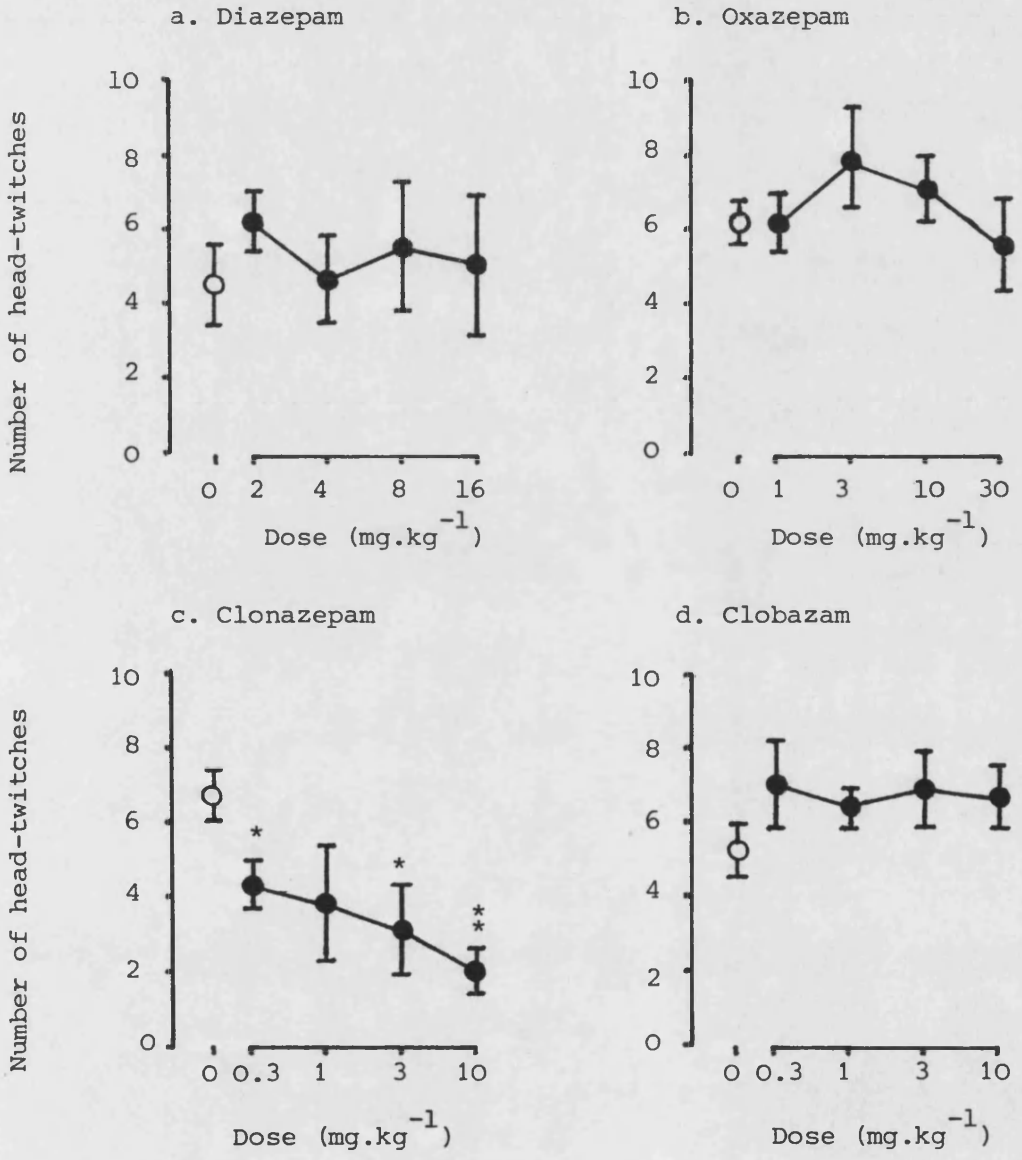


Figure 34. Inhibition by diazepam of head-twitches induced by 5-HTP (200 mg.kg^{-1}).

All values are mean ± s.e.m., n=8.

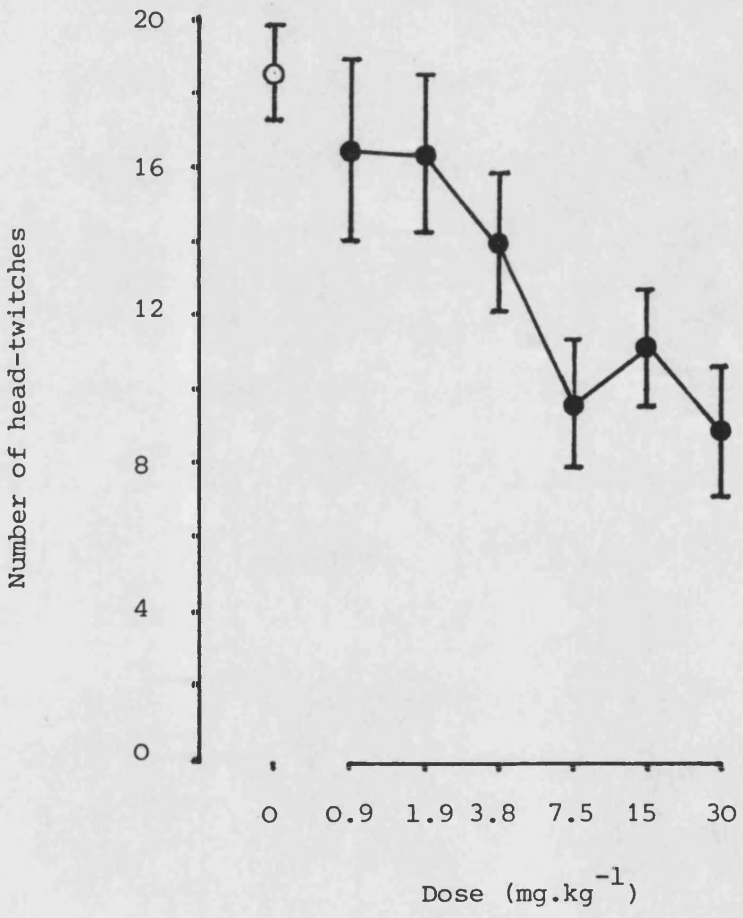


Table 8 Inhibition of 5-HTP-induced head-twitches by bezodiazepines

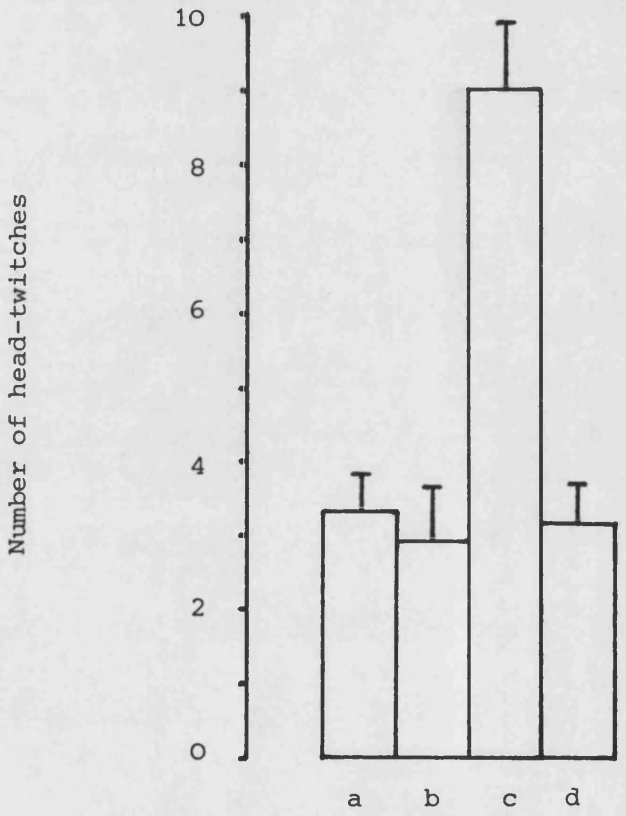
Treatment	Dose ($mg.kg^{-1}$)	n	Number of head-twitches (mean ± s.e.m.)
Vehicle	-	8	18.0 ± 2.2
Clonazepam	1	8	14.4 ± 1.5
	3	8	16.5 ± 2.3
	10	8	13.8 ± 1.3
	30	8	22.8 ± 1.8
Vehicle	-	8	16.8 ± 1.7
Oxazepam	15	8	20.0 ± 1.3
Clobazam	10	8	15.8 ± 2.4

4.4.3 Effect of Ro 15-1788 on clonazepam and 5-HTP

Treatment with the BDZ antagonist Ro 15-1788 (100 $mg.kg^{-1}$) was found to potentiate the number of head-twitches induced by a combination of clonazepam (10 $mg.kg^{-1}$) and 5-HTP (50 $mg.kg^{-1}$). These results are shown in Fig. 35.

Figure 35. Effect of Ro 15-1788 on head-twitches induced by clonazepam and 5-HTP.

All values are mean ± s.e.m., n=8.



a. 5-HTP (50 mg.kg^{-1})

b. 5-HTP (50 mg.kg^{-1}) + Clonazepam (10 mg.kg^{-1})

c. 5-HTP (50 mg.kg^{-1}) + Clonazepam (10 mg.kg^{-1}) + Ro 15-1788 (100m.kg^{-1})

d. 5-HTP (50 mg.kg^{-1}) + Ro 15-1788 (100 mg.kg^{-1})

4.5 BENZODIAZEPINES AND HEAD-TWITCHES INDUCED BY MESCALINE AND QUIPAZINE

The results presented so far, show a marked difference in the effect of BDZs on the head-twitches induced by 5-MeODMT and 5-HTP. The most obvious difference between these two 5-HT agonists is that 5-MeODMT is a directly-acting agonist whereas 5-HTP is indirectly-acting. To show that this is the important difference in the observed effects, a further two direct agonists were tested in combination with clonazepam.

4.5.1 Methods

Head-twitches were induced in mice using either quipazine (5 $mg.kg^{-1}$) or mescaline (10 $mg.kg^{-1}$) as previously described in section 2.2. Clonazepam (10 $mg.kg^{-1}$) was given 60 min. before the observation period.

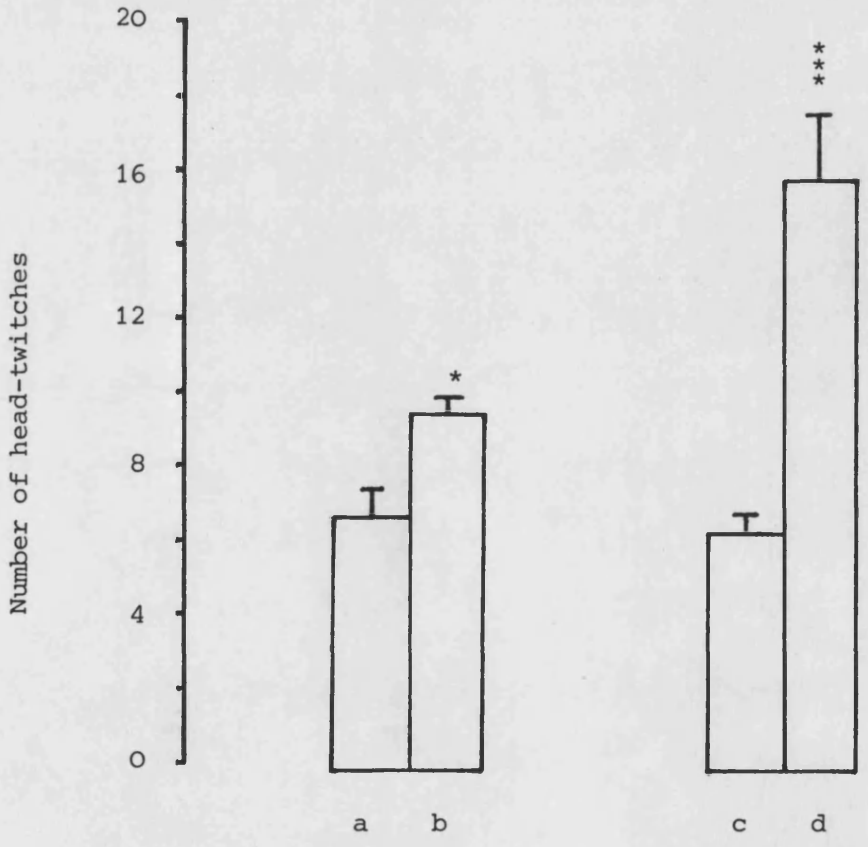
4.5.2 Results

The results are shown in Fig. 36. The number of head-twitches induced by both mescaline and quipazine was found to be significantly potentiated by pretreatment with clonazepam. The effect of clonazepam on quipazine-induced head-twitches was of a similar magnitude to that previously observed for 5-MeODMT, but the potentiation of mescaline-induced head-twitches was considerably less.

Figure 36. Potentiation of mescaline and quipazine-induced head-twitches by clonazepam

All values are mean ± s.e.m., n=6.

t-test: * $p<0.05$, *** $p<0.001$



a. Mescaline (10 $mg.kg^{-1}$)

b. Mescaline (10 $mg.kg^{-1}$) + Clonazepam (10 $mg.kg^{-1}$)

c. Quipazine (5 $mg.kg^{-1}$)

d. Quipazine (5 $mg.kg^{-1}$) + Clonazepam (10 $mg.kg^{-1}$)

4.6 EFFECT OF POTENTIATION OF GABA MECHANISMS ON THE HEAD-TWITCH RESPONSE

An involvement of GABA in the potentiation of 5-MeODMT-induced head-twitches by the BDZs seems unlikely in view of the lack of effect of bicuculline. To rule out this possibility more positively, a variety of treatments which potentiate GABA mechanisms were tested for their ability to affect the head-twitches induced by 5-MeODMT.

Muscimol acts as an agonist at the $GABA_A$ receptor (Bowery et al., 1984). This is the GABA receptor subtype to which BDZ receptors have been shown to be linked (see section 1.5). If the potentiation of head-twitches is through such a receptor, then muscimol should have the same action as the BDZs when given before 5-MeODMT. A short pretreatment time is needed with muscimol as it is rapidly metabolised by transamination (Baraldi et al., 1979).

An increase in the degree of GABA receptor stimulation was also obtained by increasing levels of GABA itself. This was achieved by using AOAA, an inhibitor of GABA transaminase, which is the main metabolic enzyme of GABA (Shusboe et al., 1974), and by inhibiting the uptake of GABA with DABA (Sutton and Simmonds, 1974).

4.6.1 Methods

Head-twitches were induced in mice using 5-MeODMT (2.5 $mg.kg^{-1}$) as previously described in section 2.2. The following treatments were tested for their effect on the head-twitch response:
Muscimol (0.5 - 2 $mg.kg^{-1}$) was administered 10 min. before the observation period; AOAA (5 - 25 $mg.kg^{-1}$) was administered 4 hours before the observation period and DABA (500 $mg.kg^{-1}$) was administered 20 hours before the observation period.

These doses and pretreatment times follow those of Menon and Vivonia (1981), Collinge et al. (1983) and Davies and Williams (1983).

The effect of muscimol was also tested against head-twitches induced by 5-HTP (50 $mg.kg^{-1}$) following carbidopa (25 $mg.kg^{-1}$) as described in section 2.2.

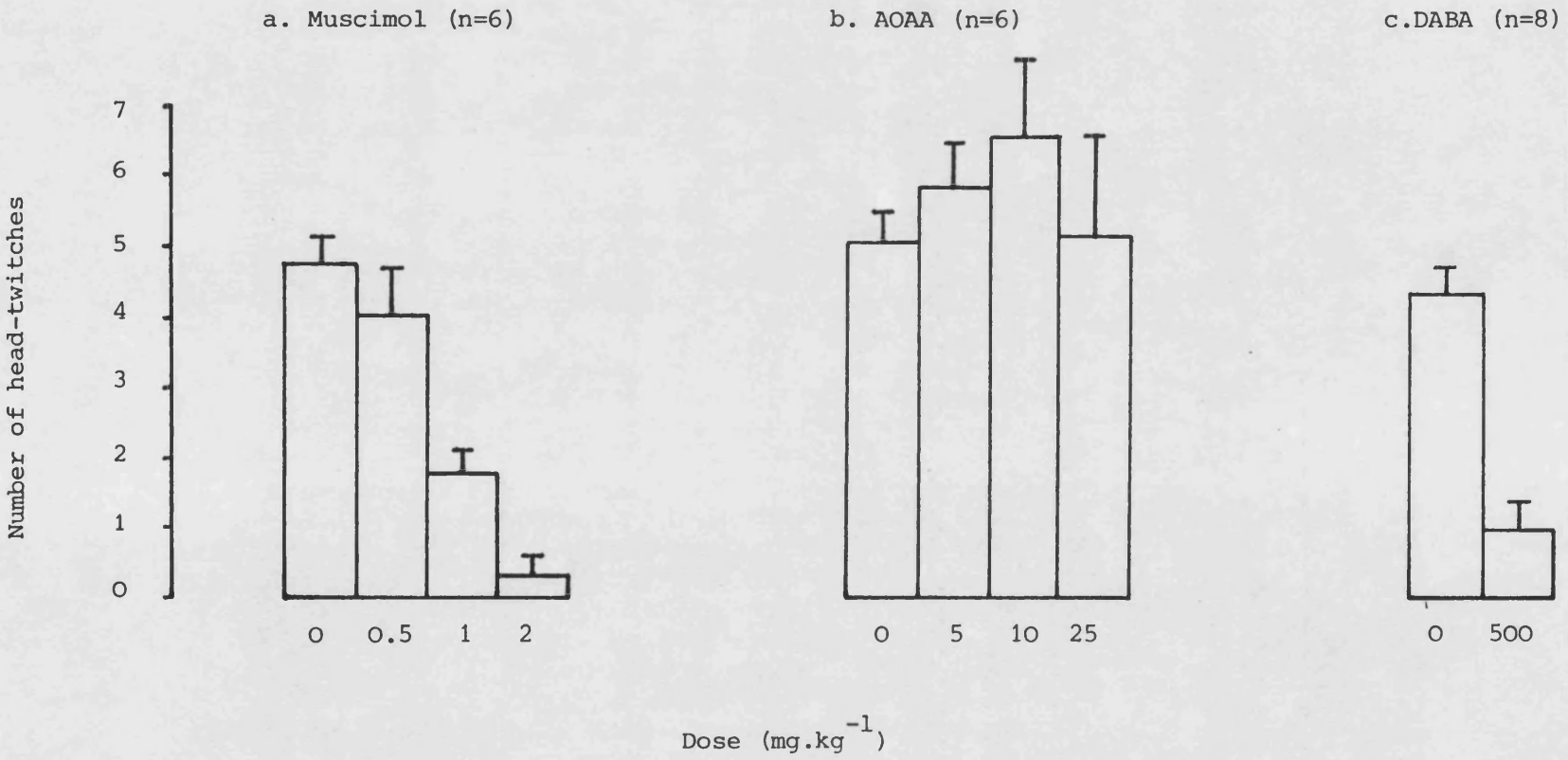
4.6.2 Results

The effect of these three treatments is shown in Fig. 37. Unlike the BDZs, none of them was found to potentiate the head-twitches induced by 5-MeODMT, and in fact the only significant effects observed were the inhibition of head-twitches following treatment with with either muscimol or DABA. The inhibition by muscimol is clearly dose related, with almost complete inhibition occurring at a dose of 2 $mg.kg^{-1}$. The doses of muscimol and of DABA that produced an inhibition of head-twitches were associated with a marked sedation, and the possibility that this was responsible for the observed inhibition cannot be ruled out. It must be remembered that the dose of 5-MeODMT used induces a very low rate of head-twitching, and results from previous experiments with the BDZs and 5-HTP-induced head-twitches have shown that a sedative dose of clonazepam can inhibit the effect of a low dose, but not a high dose, of 5-HTP.

All the doses of AOAA tested slightly potentiated the effect of 5-MeODMT, but none of these increases reached statistical significance. Like muscimol and DABA the higher doses of AOAA were associated with marked sedation, but this did not seem to affect the number of head-twiches observed.

Figure 37. Effect of drugs which potentiate GABA mechanisms on 5-MeODMT-induced head-twitches.

All values are mean ± s.e.m.



When tested against 5-HTP-induced head-twitches, the $GABA_A$ agonist muscimol was found to antagonise them in a dose dependent manner over a similar dose range to that which inhibited 5-MeODMT-induced head-twitches (Fig. 38).

4.7 THE 24-HOUR VARIATION IN THE POTENTIATION OF 5-MeODMT-INDUCED HEAD-TWITCHES BY CLONAZEPAM

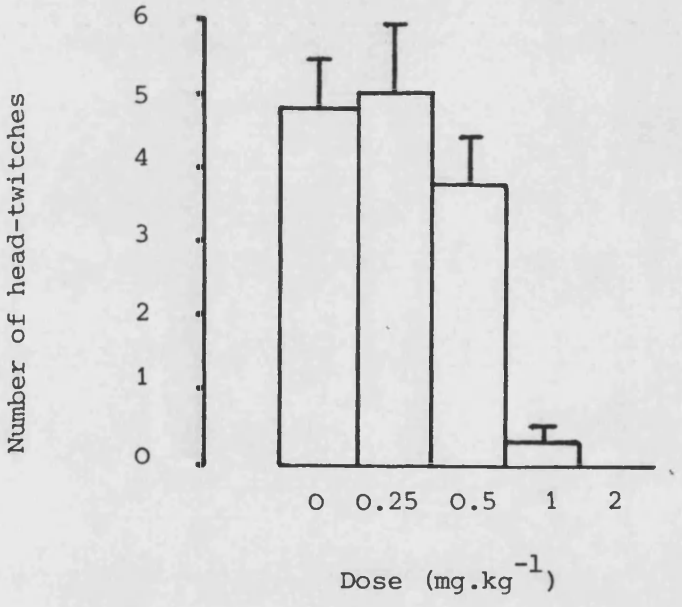
In view of the well defined 24-hour variation in the number of head-twitches induced by 5-MeODMT, it was of interest to study the potentiating effect of BDZs over 24 hours. A possible ivolvement of GABA in the organisation of circadian rhythmicity has been discussed in section 1.1.4, but an involvement of BDZs in this action of GABA has not been demonstrated. Studies of BDZ receptor binding over 24 hours, in fact, show no variation of this in the hypothalamus (Borsook et al., 1984; Brennan et al., 1985). It is not known which of the GABA receptor subtypes is present in the SCN, and they may be independent of BDZs. A 24-hour variation in numbers of BDZ binding sites has been demonstrated for other brain areas such as the frontal lobe, cerebellum and striatum (Wirz-Justice et al., 1982; Brennan et al., 1985). In the hamster, BDZs have been shown to phase shift circadian rhythms (Ralph and Mennaker, 1983).

4.7.1 Methods

Mice were housed on a 12h light-12h dark cycle as described in section 3.1, and the 24-hour variation in the potentiation of 5-MeODMT by clonazepam was studied along similar lines to the

Figure 38. Effect of muscimol on the head-twitches induced by 5-HTP.

All values are mean ± s.e.m., n=6.



experiment described in section 3.2.2.

Separate groups of 8 mice were tested at 3-hourly intervals throughout the 12h light-12h dark cycle for their response to 2.5 $mg.kg^{-1}$ 5-MeODMT following pretreatment with 5 $mg.kg^{-1}$ clonazepam or vehicle. As in previous experiments the BDZ was given 60 min. before the 3 min. observation period. The dose of clonazepam used in this experiment was chosen from the dose response curve shown in Fig. 28, as it produced an approximately half-maximal potentiation of 5-MeODMT-induced head-twitches.

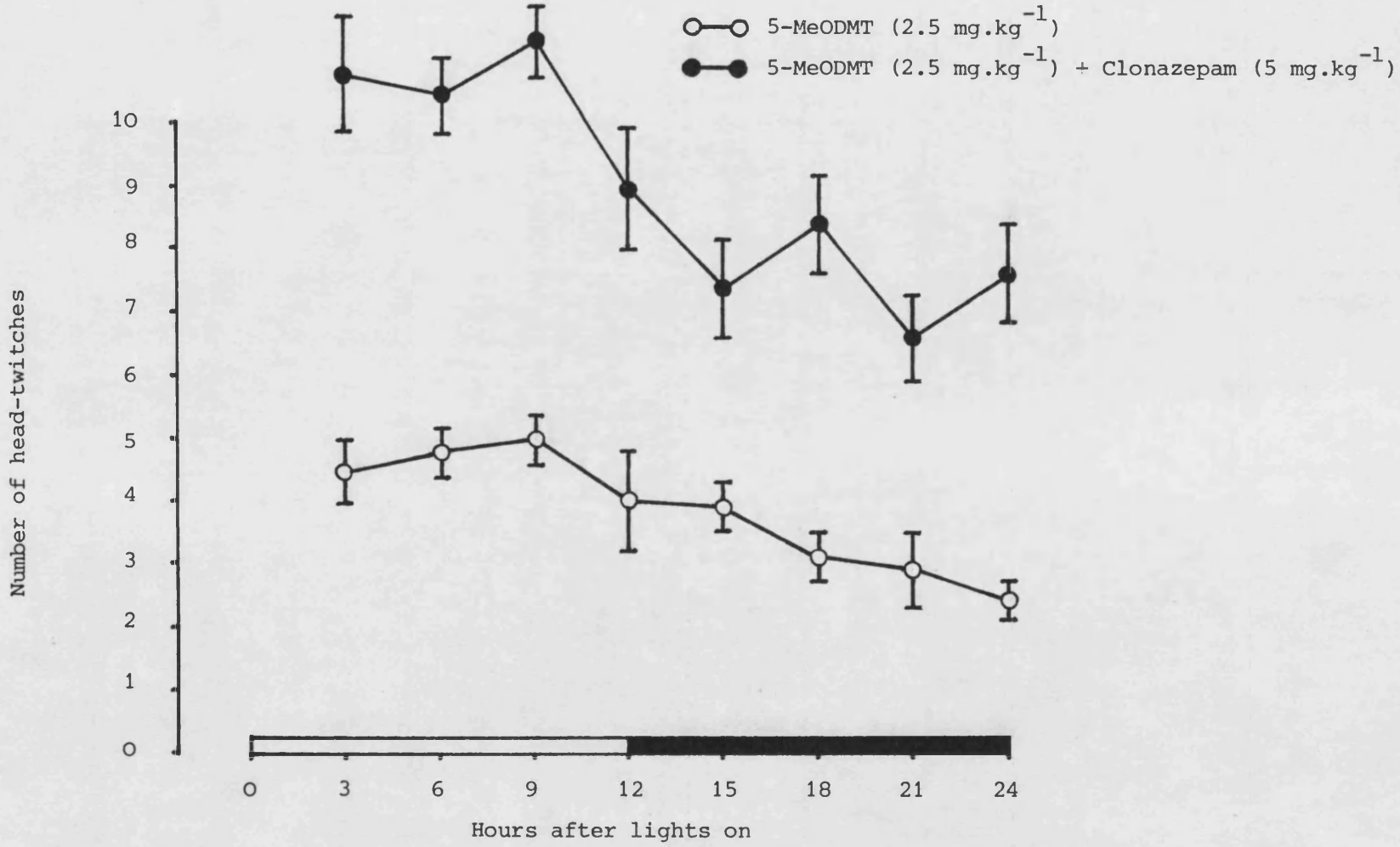
4.7.2 Results

When given to groups of mice at 3-hourly intervals throughout the light-dark cycle, the effect of 2.5 $mg.kg^{-1}$ 5-MeODMT after vehicle pretreatment was found to vary over 24 hours (Fig. 39) in a similar manner to that previously observed in response to a dose of 5$mg.kg^{-1}$ 5-MeODMT (Fig. 17). The maximum number of head-twiches was observed during the light phase, with a peak between 6 and 9 hours after the start of the light period. Analysis of variance of this data showed that the values differed significantly over the 24 hour period ($F=3.62$ for 7,56 DF, $p<0.05$). Also in agreement with the earlier results shown in Fig. 18, was the observation that the 5-HT syndrome score did not vary over 24 hours in response to 2.5 $mg.kg^{-1}$ 5-MeODMT (Table 9).

Following pretreatment with 5 $mg.kg^{-1}$ clonazepam, the 24-hour variation in head-twitch response was still present, with an apparent increase in amplitude associated with the increase in mean head-twitch response over 24 hours (Fig. 39). Again analysis of variance of the data showed that the variation of head-twitches over

Figure 39. Effect of clonazepam on the 24-hour variation of 5-MeODMT-induced head-twitches.

All values are mean ± s.e.m., n=8. Black bar represents hours of darkness.



24 hours was significant (F=4.77 for 7,56 DF, $p<0.05$). However, the percentage change in response over 24 hours is similar following either vehicle or clonazepam pretreatment. The presence of clonazepam did not affect the 5-HT syndrome response to 5-MeODMT at any of the times tested (Table 9).

Table 9 Effect of clonazepam on the 5-HT syndrome response to 2.5 $mg.kg^{-1}$ 5-MeODMT over 24 hours

Hours after lights on	Vehicle pretreatment Median (Range)	Clonazepam pretreatment Median (Range)
0	6 (3-7)	6 (3-7)
3	5.5 (5-7)	6 (4-7)
6	6 (5-8)	5.5 (3-8)
9	6 (5-7)	5.5 (5-7)
12	6 (4-7)	6.5 (3-7)
15	5 (4-7)	5.5 (4-7)
18	5 (4-7)	6 (4-8)
21	5.5 (5-7)	6 (5-6)

4.8 DISCUSSION

In agreement with the results of Nakamura and Fukushima (1977), the BDZs used in this study were found to potentiate the head-twitches induced by a variety of direct 5-HT receptor agonists. This potentiation was not due to the BDZs inducing head-twitches themselves, as only clonazepam induced significantly more head-twitches than saline pretreatment, but even this was not enough to explain its potentiation of 5-HT agonist effects. Nakamura and Fukushima (1976) reported that a number of BDZs, including some used in the experiments described in this chapter, could induce a considerable number of head-twitches when given alone, but the results of this thesis do not support this. It is therefore unlikely that the BDZs have agonist properties at the 5-HT_2 receptor, or at a receptor that has a similar effect at the same synapses. The results suggest that the BDZs are potentiating the activity of 5-HT_2 receptors in a manner analogous to their potentiating effect on the $GABA_A$ receptor complex.

This is the conclusion reached by Nakamura and Fukushima (1977, 1978b), despite their observation that BDZs themselves induced head-twitches. However, it is not possible from the results presented in this study to determine the site of the interaction. It is clearly not presynaptic as pretreatment with pCPA did not decrease the effect. The slight potentiation following pCPA, probably resulting from postsynaptic 5-HT receptor supersensitivity, and the inhibition by pirenperone of the combination of clonazepam and 5-MeODMT might indicate that the BDZs are potentiating 5-HT_2 receptor mechanisms, but it could be argued that the interaction occurs downstream of the initiating 5-HT receptors. Inhibition of these receptors would mean

that there is less activity to be potentiated at any stage between the 5-HT_2 receptor and the head-twitch response. In favour of the 5-HT_2 receptor being the site of the interaction is the observation of Nakamura and Fukushima (1976) that the 5-HT antagonist cyproheptidene was effective in antagonising the head-twitches induced by BDZs. The significance of the difference between their results and those presented here concerning induction of head-twitches by BDZs is not known. It may reflect a strain difference and indicate that the usual level of 5-HT receptor stimulation was much higher in their mice.

An involvement of GABA in the potentiation of head-twitches by the BDZs has been effectively ruled out. Pretreatment with a high dose of the $GABA_A$ antagonist bicuculline failed to inhibit it, and the use of a variety of pretreatments, that either increased GABA levels or stimulated GABA receptors, failed to mimic the effects of BDZs.

It also seems unlikely that the BDZ receptor itself is involved in the potentiation of head-twitches by BDZs. Firstly, the doses required in these experiments were generally higher than those used to demonstrate anxiolytic, antiepileptic, muscle relaxant and sedative properties of BDZs in animals (e.g. Thiebot et al., 1980). Secondly, and more importantly, it was not possible to antagonise the effects of BDZs with either the BDZ receptor antagonist Ro 15-1788, in agreement with Nakamura and Carney (1983), nor with the inverse agonist β-CCE. These compounds did appear to reverse the sedative actions of clonazepam, showing that they could inhibit effects thought to depend on BDZ receptor stimulation.

The BDZs would therefore appear to be potentiating postsynaptic

5-HT_2 receptor actions by an as yet uncharacterised mechanism. It is tempting to speculate that a receptor site, at which BDZs are weak agonists, is linked to the 5-HT_2 receptor. Activation of this receptor would potentiate the activity of the 5-HT receptor, but have no effect itself. There is no direct evidence for this at present, and merely because BDZs have this action is no reason to draw comparisons with the $GABA_A$ receptor complex.

In complete contrast to the effects of BDZs on the action of direct 5-HT_2 receptor agonists, no potentiation of the head-twitches induced by 5-HTP was observed. Like the head-twitches induced by 5-MeODMT, there is good evidence that these are mediated by the 5-HT_2 receptor (see section 2.2). Handley and Singh (1984b, 1985) reported that $GABA_A$ mechanisms potentiated 5-HTP-induced head-twitches, and that $GABA_B$ mechanisms inhibited them. It was therefore surprising that the BDZs, which potentiate $GABA_A$ mechanisms, did not also potentiate 5-HTP-induced head-twitches. It is possible that the $GABA_A$ receptors involved in this response were not stimulated enough, but the results presented here point to an alternative explanation, particularly as muscimol was not found to potentiate head-twitches, as had been reported by Handley and Singh (1985).

Following pretreatment with 5-HTP it is necessary for this compound to be decarboxylated to 5-HT before it can act on 5-HT receptors. This process presumably requires that 5-HTP enters the nerve terminal where it is converted to 5-HT and then is either released by the normal process, or 'spills out' of the nerve terminal into the synaptic cleft. As discussed in section 1.5, the BDZs have been shown to decrease 5-HT neuronal activity by potentiation of a GABA-mediated presynaptic inhibition. It might be

expected that this decrease in presynaptic activity would reduce the activity of 5-HTP as less of the 5-HT formed would be released to act postsynaptically. This is the explanation used by Metz et al. (1985) to explain the inhibition of 5-HTP-induced head-twitches by the $GABA_B$ receptor agonist baclofen. Schlicker et al. (1984) had already shown that $GABA_B$ mechanisms could inhibit 5-HT release in vitro, and the results of Metz et al. (1985) suggest that this can occur in vivo.

The results obtained with the BDZ antagonist Ro 15-1788 against the head-twitches induced by 5-HTP and clonazepam would support this interpretation. Following Ro 15-1788 pretreatment, clonazepam was found to potentiate 5-HTP-induced head-twitches. This result can be interpreted as an inhibition of the presynaptic effects of BDZs, resulting in the release, by normal processes, of 5-HT formed from administered 5-HTP, which is then potentiated just like the effects of the direct 5-HT receptor agonists.

From these results it would seem that under normal circumstances the presynaptic effects of BDZs on 5-HT neuronal activity predominate over the postsynaptic effects. This makes it unlikely that the postsynaptic potentiation is responsible for any of the clinical effects of BDZs. However, it may be of relevance for drug interactions of the BDZs. Furthermore, the site of action of the BDZs in potentiating $5\text{-}HT_2$ receptor activity may provide a new site for drug activity, if specific agonists could be developed.

These results also highlight the importance of using a variety of 5-HT agonist to ensure that the pre- and postsynaptic actions against them can be detected.

In view of the above conclusions it is difficult to evaluate the

significance of the lack of 24-hour variation in clonazepam potentiation of 5-MeODMT head-twitches. Clearly the 24 hour rhythms previously observed for BDZ binding sites (Brennan et al., 1985; Wirz-Justice et al., 1982) will have little relevance to these results, as it does not appear to be a BDZ receptor that is involved. If the potentiation of head-twitches by BDZs represents an action at a functional receptor, these results indicate that this receptor does not show a 24-hour variation and is therefore not responsible for the 24-hour variation in head-twitch activity. It may thus represent a control mechanism for the 5-HT_2 system. The linking of a receptor which changes activity over 24 hours with one that does not, may allow more variety and greater control of the functional activity of that system. There is, however, no evidence to indicate that this site of action of BDZs represents a functionally important receptor.

5 EFFECT OF ANTICHOLINESTERASES ON 5-HT RECEPTOR-MEDIATED BEHAVIOURS

5.1 INTRODUCTION

In addition to the effects of benzodiazepines, which have been reported in the previous chapter, the effects of potentiation of cholinergic transmission on 5-HT receptor mediated behaviours was also studied. Details of what is known about acetylcholine (ACh)-5-HT interactions have already been discussed in section 1.6. The increased 5-HT turnover following inhibition of acetylcholinesterase (Fernando et al., 1984) leads one to expect that a potentiation of 5-HT receptor-mediated behaviours may be observed, particularly as benzodiazepines, which decrease 5-HT turnover, were found to inhibit 5-HTP-induced head-twitches.

The results of Fernando et al. (1984) demonstrated an interaction of ACh and 5-HT in the striatum, and these interactions were found to affect motor responses. It was therefore decided to study the effects of anticholinesterases on the head-twitch and 5-HT syndrome induced by 5-MeODMT and 5-HTP, and also on the hypermotility induced by RU 24969. Further details of these behaviours can be found in section 1.4 and chapter 2.

In all the experiments reported in this chapter, the ACh-5-HT interaction has been studied by increasing levels of ACh using reversible anticholinesterases. The main compound studied was physostigmine (eserine); neostigmine was also used in some experiments. The action of these drugs is to inhibit the activity of the enzyme acetylcholinesterase, which is the primary mechanism for the termination of action of ACh. They therefore greatly increase ACh concentrations in cholinergic synapses. As cholinergic neurones are widespread throughout the CNS and the periphery, anticholinesterases are highly toxic, and their most

extensive practical application has been as insecticides and as chemical warfare nerve gases (Koelle, 1975).

Physostigmine is an alkaloid obtained from the Calabar bean, the dried ripe seed of Physostigma venenosum Balfour, a woody climber found in tropical West Africa. The pure alkaloid was first isolated from the Calabar bean in 1864 by Jobst and Hesse, and named physostigmine. Its first therapeutic use was in 1877 for the treatment of glaucoma, for which it is still used today. A full account of its history has been given by Holmstedt (1972).

Neostigmine was first synthesised in 1931 by Aeschlimann and Reinert as a result of the work of Stedman (1929) elucidating the mechanism of action of physostigmine. It was reported to be effective in the treatment of myasthenia gravis (Remen, 1932; Walker, 1935) and is still used to treat this disease. Neostigmine is a substituted phenyl ester of carbamic acid and reacts with acetylcholinesterase in a manner similar to physostigmine. Once these compounds have reacted with the enzyme, hydrolysis back to its active state is much slower than following ACh cleavage. It is, however, a reversible, if slow process, unlike the organophosphate anticholinesterases which are essentially irreversible.

Inhibitors of acetylcholinesterase are one of the few classes of drugs for which the mechanism of action can be described in precise chemical terms. For those interested, a description of the processes involved in the cleavage of ACh into acetic acid and choline, and in the actions of reversible and irreversible inhibitors of the enzyme, has been given by Koelle (1975).

5.2 METHODS

5.2.1 Animals

In all experiments reported in this chapter, female CFLP mice (25-40g) were used. They were housed in the stock rooms of the University of Bath animal house on a 14h light-10h dark cycle, with lights on at 0500 hours. Food and water were freely available at all times.

It was necessary to use female mice for this part of the study due to a shortage of male mice in the animal house.

5.2.2 Drugs

Details of dose volume, routes of administration and vehicles used have already been given for many of the drugs used in this chapter in section 2.1. All compounds were dissolved or suspended in 0.9% saline, and injected i.p..

The drugs used in these experiments, in addition to those already mentioned, were: physostigmine salicylate (Sigma); neostigmine (Sigma); (-)-scopolamine HCl (Sigma).

5.3 5-HT AGONIST-INDUCED HEAD-TWITCH AND SYNDROME IN FEMALE MICE

As all previous experiments had been performed using male mice, it was necessary to ensure that female mice responded in a similar manner to the 5-HT agonists 5-MeODMT and 5-HTP.

5.3.1 Methods

The methods used to induce head-twitches and the 5-HT syndrome in female mice using 5-MeODMT and 5-HTP were exactly as previously described for male mice in section 2.2.

5.3.2 Results

The dose response curves for 5-MeODMT and 5-HTP-induced head-twitch and syndrome are shown in Fig. 40. It can be seen that the responses are similar to those observed in male mice (Figs. 2 and 3). In view of this, the same doses were used in female mice as had already been used in male mice to examine inhibition and potentiation of these behaviours. These were 16 and 2.5 $mg.kg^{-1}$ respectively for 5-MeODMT, and 200 and 50 $mg.kg^{-1}$ for 5-HTP.

5.4 EFFECT OF ANTICHOLINESTERASES ON HEAD-TWITCH AND SYNDROME RESPONSE

All anticholinesterases and scopolamine were given 30 min. before the observation period for head-twitches.

5.4.1 Effect of physostigmine on low doses of 5-MeODMT and 5-HTP

When given before 2.5 $mg.kg^{-1}$ 5-MeODMT, sub-toxic doses of physostigmine had no effect on either the head-twitch response, or on the 5-HT syndrome response (Table 10).

Figure 40. Dose-response curves for 5-MeODMT and 5-HTP in mice.

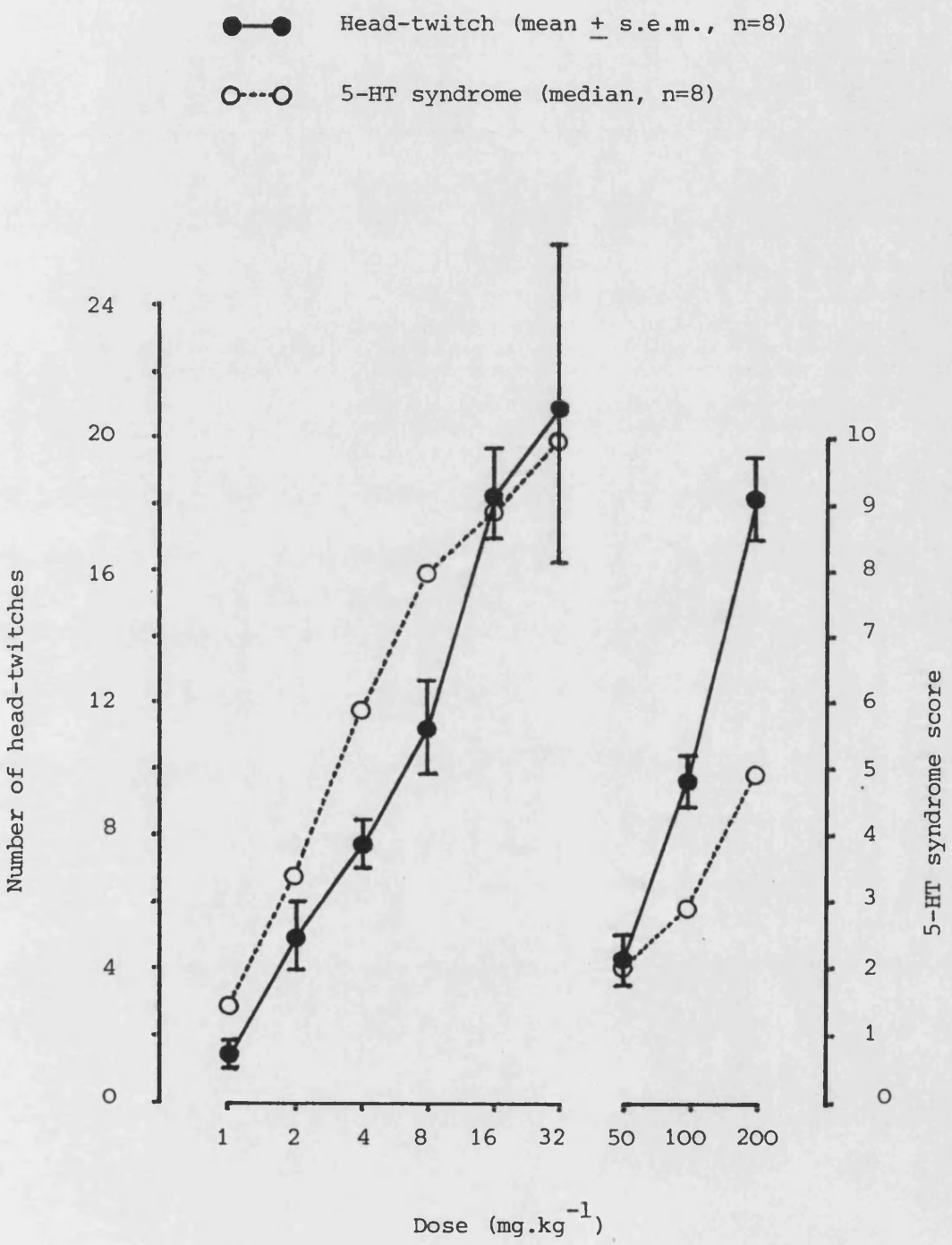


Table 10 Effect of physostigmine on 2.5mg.kg^{-1} 5-MeODMT

Treatment	Dose (mg.kg^{-1})	n	Head-twitches (mean ± s.e.m.)	5-HT Syndrome (Median and range)
Saline	-	8	4.5 ± 0.4	5 (3-7)
Physostigmine	0.1	8	4.4 ± 0.5	5.5 (4-7)
	0.2	8	3.9 ± 0.6	5 (4-6)

The effect of physostigmine on 50 mg.kg^{-1} 5-HTP is shown in Table 11. Unlike its effect on 5-MeODMT, physostigmine was found to significantly inhibit the head-twitches induced by 5-HTP.

Table 11 Effect of physostigmine on 50 mg.kg^{-1} 5-HTP

Treatment	Dose (mg.kg^{-1})	n	Head-twitches (mean ± s.e.m.)	t-test (v. saline)
Saline	-	6	5.5 ± 1.1	
Physostigmine	0.1	6	2.0 ± 0.7	$p<0.05$
	0.2	6	1.5 ± 0.4	$p<0.01$
	0.4	6	1.8 ± 1.3	ns

5.4.2 Effect of anticholinesterases on high doses of 5-MeODMT and 5-HTP

The effect of physostigmine on the head-twitches induced by 5-MeODMT (16 $mg.kg^{-1}$) or 5-HTP (200 $mg.kg^{-1}$) is shown in Fig. 41. Higher doses of physostigmine could not be tested as at doses above 0.4 $mg.kg^{-1}$ the mice did not survive the course of the experiment. Despite this, it was clear that sub-toxic doses of physostigmine significantly inhibited the head-twitches induced by both 5-MeODMT and 5-HTP in a dose related fashion.

The effect of neostigmine on head-twitches induced by 5-MeODMT is shown in Fig. 42, and like physostigmine it too was found to significantly inhibit head-twitches in a dose-dependent manner at sub-toxic doses.

The data in Table 12 shows how physostigmine and neostigmine affected the 5-HT syndrome induced by 5-MeODMT. Unlike the 5-MeODMT-induced head-twitches, neither of the anticholinesterases significantly inhibited the 5-HT syndrome (Mann-Whitney U test).

The effect of physostigmine on the 5-HT syndrome induced by 5-HTP (200 $mg.kg^{-1}$) is shown in Table 13. Lower scores are obtained for the 5-HT syndrome when it is induced by 5-HTP, and at the highest dose of physostigmine used a significant inhibition of the syndrome was recorded.

Figure 41. Effect of physostigmine on head-twitches induced by 5-MeODMT and 5-HTP.

All values are mean ± s.e.m., n=6.

t-test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

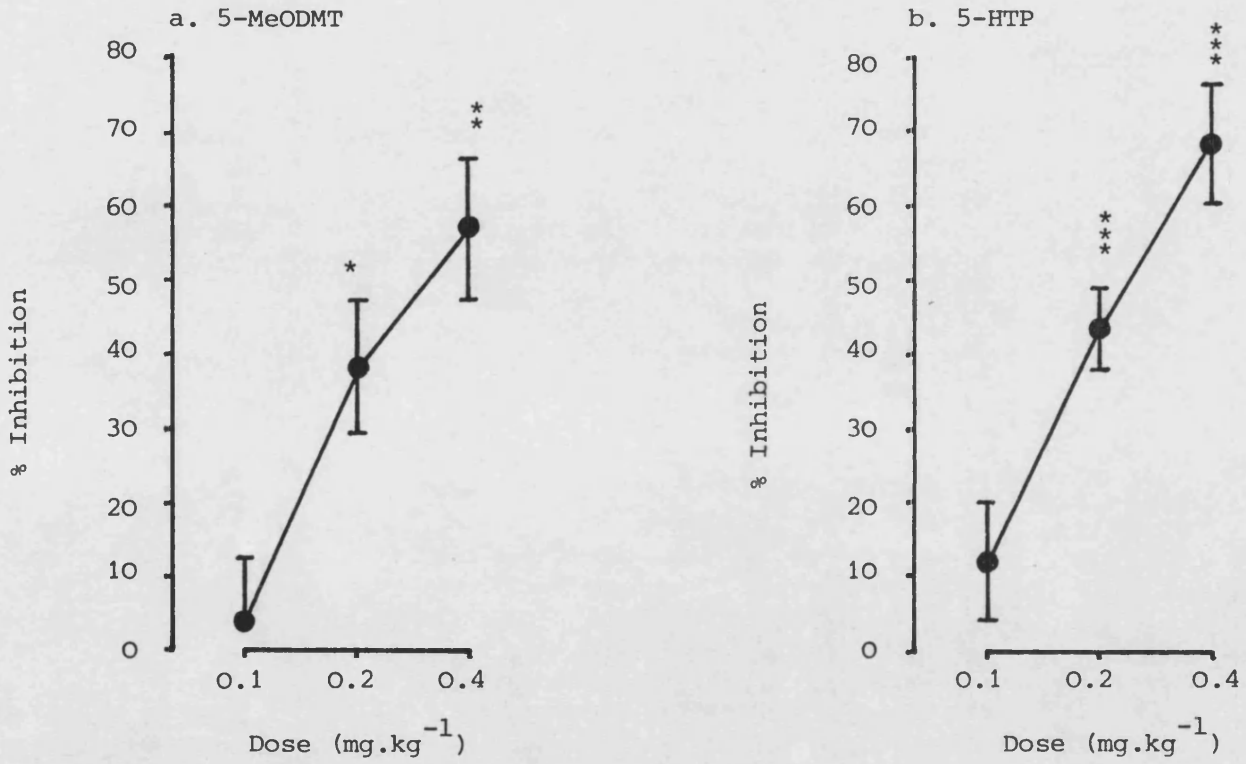


Figure 42. Effect of neostigmine on head-twitches induced by 5-MeODMT.

All values are mean ± s.e.m., n=6.

t-test: *** p<0.001

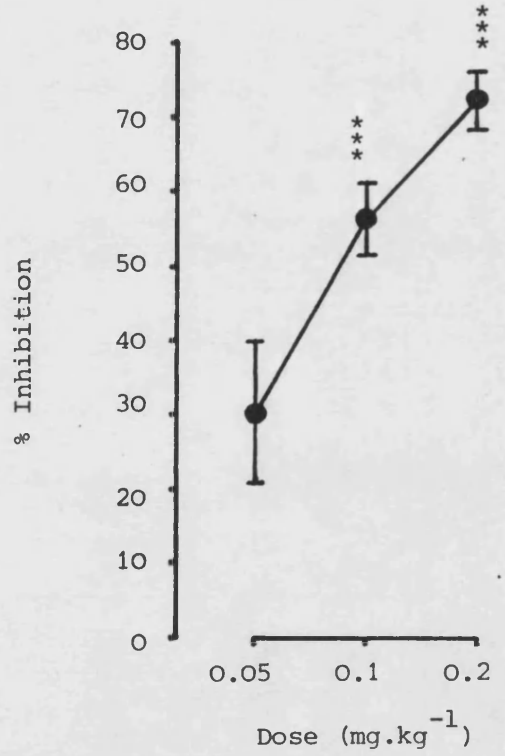


Table 12 Effect of anticholinesterases on 5-MeODMT-induced 5-HT syndrome

Treatment	Dose ($mg.kg^{-1}$)	n	5-HT syndrome (Median and range)
Saline	-	6	9 (8-10)
Physostigmine	0.1	6	9 (8-10)
	0.2	6	8 (7-10)
	0.4	6	9 (7-10)
Saline	-	6	9.5 (8-10)
Neostigmine	0.05	6	8.5 (8-10)
	0.1	6	7.5 (6-9)
	0.2	6	9 (7-9)

Table 13 Effect of physostigmine on the 5-HTP-induced 5-HT syndrome

Treatment	Dose ($mg.kg^{-1}$)	n	5-HT syndrome (Median and range)	Mann-Whitney U-test
Saline	-	6	5 (4-7)	
Physostigmine	0.1	6	4.5 (3-7)	
	0.2	6	4 (2-5)	
	0.4	6	1.5 (1-3)	$p<0.01$

5.4.3 Inhibition of anticholinesterase effects by scopolamine

To show that the effects of physostigmine and neostigmine resulted from increased ACh concentrations in the synapse, they were tested in combination with scopolamine. This is a muscarinic receptor antagonist, and was used in preference to atropine because of its better penetration into the CNS (Innes and Nickerson, 1975).

Scopolamine (4 $mg.kg^{-1}$) was found to reverse the effects of physostigmine (0.4 $mg.kg^{-1}$) and neostigmine (0.2 $mg.kg^{-1}$) on the head-twitches induced by 5-MeODMT, as shown in Fig. 43. This dose of scopolamine alone had no effect on 5-MeODMT-induced head-twitches (Fig. 43).

5.5 EFFECT OF PHYSOSTIGMINE ON RU 24969-INDUCED HYPERACTIVITY

5.5.1 Methods

Hyperactivity was induced in female mice using RU 24969 using the same method as that previously described in section 2.4 for male mice.

The effects of physostigmine (0.4 $mg.kg^{-1}$) and scopolamine (4 $mg.kg^{-1}$) were examined on the hyperactivity induced by RU 24969 (1.25 $mg.kg^{-1}$).

5.5.2 Results

The results are shown in Fig. 44. Physostigmine was found to inhibit locomotor activity in both saline and RU 24969 pretreated mice, in both cases by approximately 50%. The effect of scopolamine is shown in Fig. 45. Like physostigmine, scopolamine was found to

Figure 43. Reversal of the effects of anticholinesterases on 5-MeODMT-induced head-twitches by scopolamine.

All values are mean ± s.e.m., n=6.

M 5-MeODMT (16 $mg.kg^{-1}$)

P Physostigmine (0.4 $mg.kg^{-1}$)

N Neostigmine (0.2 $mg.kg^{-1}$)

S Scopolamine (4 $mg.kg^{-1}$)

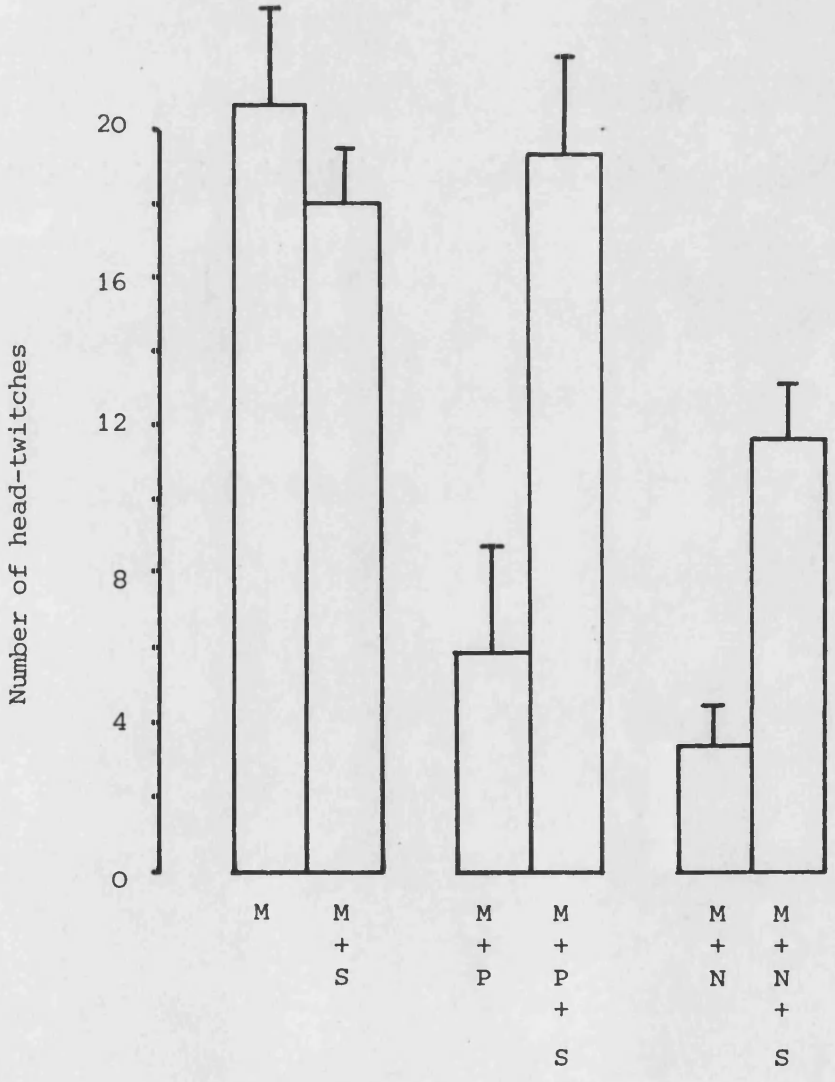
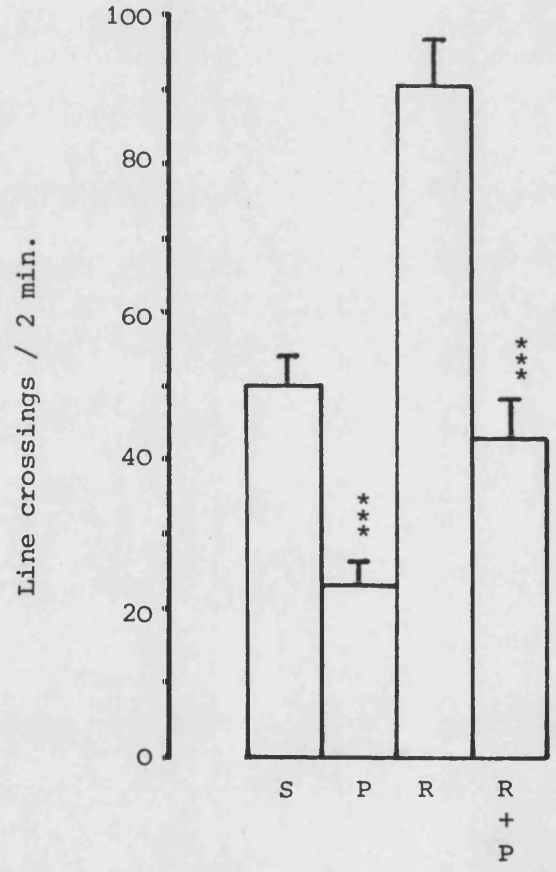


Figure 44. Effect of physostigmine on RU 24969-induced hyperactivity.

All values are mean ± s.e.m., n=6.

t-test (a v. b and c v. d) *** $p<0.001$



S Saline

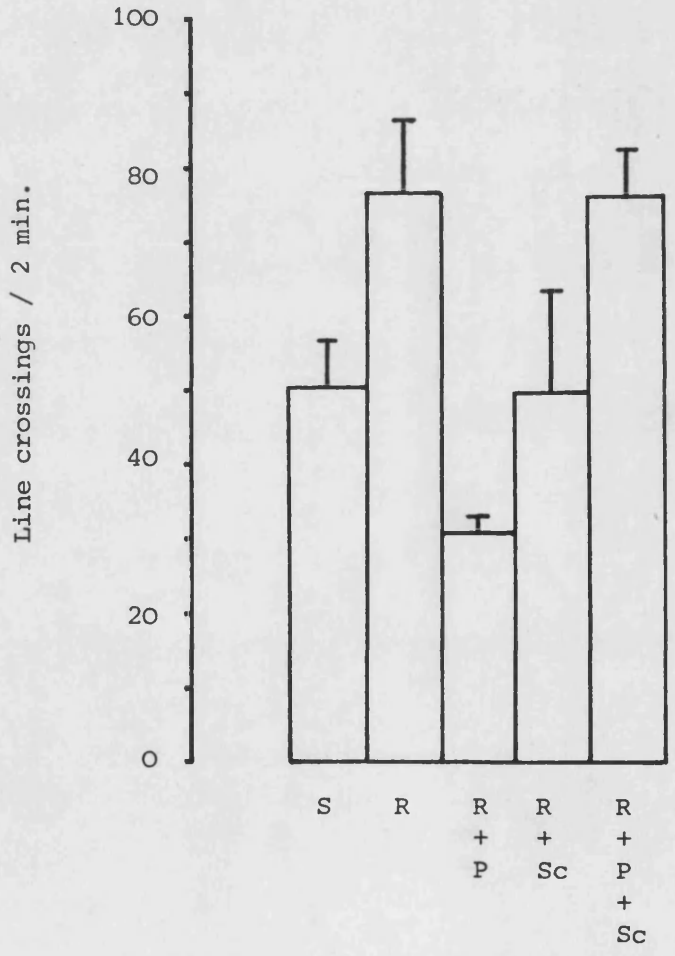
P Physostigmine (0.4 $mg.kg^{-1}$)

R RU 24969 (1.25 $mg.kg^{-1}$)

Figure 45. Reversal of physostigmine inhibition of RU 24969-induced hyperactivity by scopolamine.

All values are mean ± s.e.m., n=6.

S Saline
R RU 24969 (1.25 $mg.kg^{-1}$)
P Physostigmine (0.4 $mg.kg^{-1}$)
Sc Scopolamine (4 $mg.kg^{-1}$)



inhibit the locomotor activity induced by RU 24969, although insignificantly, but when given in combination with physostigmine it reversed the inhibition of locomotor activity. The animals that had received physostigmine exhibited a persistent tremor and hind-limb abduction in all experiments. This may have contributed to the observed effects.

5.6 DISCUSSION

The experiments reported in this chapter are only a preliminary study, and some of the results obtained clearly warrant further investigation. Despite this, it is possible to draw some tentative conclusions.

The observation that all the effects of anticholinesterases demonstrated in these experiments can be reversed by simultaneous administration of scopolamine indicates that they are the result of increasing ACh levels at muscarinic receptors. However, it is not clear to what extent these results represent a physiological interaction. As indicated in the introduction, previous experiments have shown that ACh and 5-HT enhance each others function. This is clearly not the case in these experiments, except perhaps for the effect of scopolamine on RU 24969-induced hyperactivity. This effect does not reach statistical significance, but may nevertheless be physiologically relevant. It is interesting that the administration of physostigmine and scopolamine together fails to inhibit RU 24969-induced hyperactivity which they both inhibit when given alone. This

would imply that different sites of action are responsible for these effects, although further experiments would be needed to confirm this. The simplest explanation, and perhaps the most likely, is that the effects of physostigmine on locomotor activity are manifestations of its toxicity. At the dose used in this experiment, physostigmine-treated animals showed moderate tremor and hind-limb abduction. These behaviours would severely inhibit the ability of the animal to ambulate normally. The inhibition by scopolamine of RU 24969-induced hyperactivity is in agreement with the suggestion that ACh and 5-HT facilitate each others action (Fernando et al., 1984; Ladinsky et al., 1981). It is possible that the toxic effects of physostigmine are masking other effects that may be present.

Despite the fact that physostigmine induced tremor and hind-limb abduction, there was no evidence that these particular behaviours, which are part of the 5-HT syndrome, were more or less affected than the other aspects of the 5-HT syndrome induced by 5-MeODMT or 5-HTP. It was noticable, however, that the 5-HT syndrome was considerably less affected than the head-twitch response induced by these agents. The only time an inhibition of the syndrome was observed was with the highest dose of physostigmine against 5-HTP. The syndrome induced by 5-HTP was of a much lower intensity than that induced by 5-MeODMT (as evidenced by the control scores) and this may be the reason for the inhibition. It is difficult to know how much importance to attach to this result.

In contrast to the 5-HT syndrome, the head-twitch induced by both 5-HTP and 5-MeODMT was antagonised by pretreatment with anti-cholinesterases. Like their action on RU 24969-induced hyperactivity, this was reversed by scopolamine. Also like the

effect of anticholinesterases on RU 24969-induced hyperactivity, it is difficult to conclude that this is anything other than an effect due to their toxicity. This is because the effect disappeared very rapidly as the dose was reduced, and the head-twitch response, like locomotor activity, requires some coordination of muscle movements, unlike most aspects of the 5-HT syndrome. It is possible that there is a functional difference represented in the effects of the anticholinesterases on the head-twitch and syndrome responses, but more experiments would be required to demonstrate this conclusively.

6. GENERAL DISCUSSION

6. GENERAL DISCUSSION

The results presented in this thesis have already been extensively discussed in each of the relevant chapters. It is the purpose of this discussion to provide some general comment on the methods used, and to suggest areas where further investigation would be particularly beneficial.

6.1 24-hour variation

The main bulk of this thesis has been concerned with behaviours mediated by subtypes of the 5-HT receptor, and their activity over a 12h light - 12h dark period. The identification and characterisation of 5-HT receptors, and of behaviours mediated by them, are areas that are still being extensively studied and about which there is still considerable controversy. Because of this, many of the conclusions that have been drawn are necessarily tentative. It is possible that much of this thesis will have to be reinterpreted within a short space of time, such appears to be the rate of progress in this field at present. Glennon (1986) has described 5-HT as entering a second childhood, and written that new questions are rapidly outpacing new answers. It is difficult to disagree with this statement at present.

From the results described in this thesis it has been concluded that behaviours depending on 5-HT_2 receptors for their initiation show a 24-hour variation, whereas those that depend on 5-HT_1 receptors do not. The variation in the head-twitch response has been demonstrated in a number of separate experiments, and in all cases the peak of the variation has been between 6 and 9 hours after lights on. A number of possible reasons for this difference have

already been discussed in chapter 3. A number of more general considerations will now be discussed, some of which also have relevance to the other experiments reported in this thesis.

It is immediately noticeable that it is what is probably the best characterised of the models examined that has shown a 24-hour variation. This lack of precise classification of the other behaviours may merely be a result of the lack of specific pharmacological agents available to study them, a point often raised in previous discussions. However, it could also be part of the reason why behaviours other than the head-twitch failed to display a 24-hour variation. The fact that they have eluded precise classification could be because they are far more complex and involve a large number of transmitter systems in their expression. It is well known, for example, that control of body temperature (e.g. Milton, 1978) and locomotor activity (e.g. Kelly, 1977) in particular,are open to a wide variety of influences. The interaction of a number of systems in behaviours such as these could lead to a damping of any 24-hour variation present in the initiating receptors.

There are other possible reasons why a variation should be detected in one model, rather than others, that are also independent of whether or not the initiating receptors show a 24-hour rhythm. One of these is the problem of 24-hour variation in observer performance, and the effect this will have on the introduction of unintentional bias into the results. The head-twitch response is probably the most likely behaviour to suffer from changes in observer performance, as it is by far the most transient of those studied. It is hoped that any effects this may have on the results have been

minimised. It has already been mentioned that groups of animals were phase shifted to make experiments more convenient to carry out. This procedure effectively breaks up the testing into a non-consecutive sequence, and thus pseudo-randomises the order of testing. Furthermore, in those experiments where only responses at mid-light and mid-dark were considered , the two groups were studied at the same time. This makes it unlikely that changes in observer performance were responsible for the variation in head-twitch activity.

Observer bias, on the other hand, is far more difficult to eradicate. Ideally, all experiments should be performed with the observer unaware of either the treatments received by the animals, or any expectations of the results, however vague these may be. Unfortunately, it was not possible to meet these criteria for most of the experiments reported. In many cases, all animals received the same treatment, and it would be almost impossible for a single experimenter to remain unaware of the time of testing. Some of the experiments studying BDZ-5-HT interactions were carried out 'blind', with the treatments identified only by letter. This procedure was of limited value, however, as many compounds used were insoluble in the vehicles used, thus providing clues as to the likely identity of each treatment.

The problem of experimenter bias has been studied in detail for T-maze procedures by Rosenthal and Fode (1963). They found that experimenter expectations of animal performance could significantly affect the actual performance of the animal in learning a T-maze discrimination task. They suggested that the ways in which information could be transmitted to the animal varied from the

relatively gross, such as the way in which an animal was removed from the maze, to the much more subtle, such as changes in skin temperature or moisture. We can only guess at what an animal makes of the many signals it receives from an experimenter, and it would be very brave to suggest that such effects can be discounted from the results presented here. This problem is particularly acute in behavioural experiments, and while every attempt was made to be consistent in such things as animal handling, dosing, conditions of testing etc., it is difficult to be certain that all relevant variables have been controlled.

Another question mark that must be raised against the conclusions reached in this thesis concerns the validity of using drug-induced behaviours to draw inferences about the activity of the receptors involved. All of the behaviours studied in this thesis represent an artificially high level of stimulation of the receptors involved. Under such circumstances it is not certain that all the systems usually operating to control the activity of the receptors involved are functioning normally. Not only that, but 24-hour variations that are not normally important may become relevant. For example, 5-HT uptake may not normally be a contributing factor in controlling 5-HT concentrations in the synapse, even though it may show a variation in its activity. When the 5-HT system is overloaded with 5-HT, such as when a high dose of 5-HTP is administered to an animal, the variation in 5-HT uptake may then become important. There is no way to control for this except by studying a system in a number of ways. This is one reason why it was important to test a number of 5-HT agonists for induction of the head-twitch response. It must also remain a possibility that this introduction of factors

not normally important could damp out a 24-hour variation.

All of the factors discussed above will undoubtably have played a part in the results of this thesis. While it is not considered that they are the prime variables, they should be born in mind when further experiments are both planned and interpreted. It is thus important to follow up the behavioural data with experiments that suffer from a different set of undesirable influences. Some of these could study 5-HT receptor activity more specifically and directly. This task will be considerably easier when the current confusion surrounding central 5-HT receptor subtypes has been settled, and with the development of more specific agonists and antagonists for these receptors.

A technique that deserves further use to study 24-hour variation of 5-HT receptor activity is that of ligand binding. The receptor binding work carried out over 24 hours is not conclusive, as already discussed in chapter 3. Receptor binding studies using some of the more recently introduced ligands for specific 5-HT receptor subtypes, such as ketanserin and 8-OHDPAT, could be particularly useful. However, in binding experiments the functional activity of the binding sites cannot be assessed. If the 24 hour variation in activity of the 5-HT_2 receptor is as a result of decoupling between the receptor site and the second messenger system, receptor binding would not detect a change. Studies of the 5-HT receptor-stimulated second messenger systems would circumvent this problem. Despite the problems with attribution of second messenger systems to 5-HT receptor subtypes (see chapter 1), experiments of this type would undoubtably complement the results of this thesis, and may shed further light on the mechanisms involved in the 24-hour

variation of head-twitch activity and lack of such variation in the other behaviours studied.

It would also be of interest to study other models for the 5-HT_2 receptor. The most obvious choice would be the use of LSD as a discriminative cue, which appears to be mediated by the 5-HT_2 receptor (see section 2.3). This would have the added advantage of complementing the 5-HTP-saline discrimination study carried out over 24 hours.

6.2 Interactions of benzodiazepines with 5-HT

Many of the comments in the previous section could equally well apply to these experiments, and will not be further discussed. The most interesting feature to arise from these experiments is the possibility of a receptor site linked to the 5-HT_2 receptor. This is clearly deserving of further investigation, and perhaps the best way at first would be to search for antagonists of this effect. This could be done by looking for agents which antagonised the head-twitches induced by a BDZ-5-MeODMT combination, but not those induced by 5-MeODMT alone. If such an agent were found it would confirm the site of the interaction as being on the postsynaptic neurone at 5-HT synapses. An alternative method to investigate the site of the interaction could be the use of 5-HT-stimulated second messenger systems, such as inositol phospholipid breakdown (see section 1.3). The effects of BDZs on binding of ligands for the 5-HT_2 site may also produce some interesting results.

An area that has not been studied at all in this thesis is the effect of BDZs on behaviours mediated by the 5-HT_1 receptor. It has recently been shown that chronic treatment with clonazepam can

induce an increase in the number of 5-HT_1 binding sites in rat frontal cortex (Wagner et al., 1985), showing that it is possible for BDZs to affect this 5-HT receptor as well. It is interesting that diazepam did not produce similar changes in their experiments. This suggests that this response, like the effect of BDZs on head-twitches, may not depend on activation of BDZ receptors. Much of the work reported here on the head-twitch response and BDZs could be repeated using those models thought to depend on 5-HT_1 receptor activation. It would also be of interest to determine the effects of chronic treatment with BDZs on the head-twitch response, as the pre- and postsynaptic effects they have on 5-HT neurones may respond differently. This could have implications for the clinical use of BDZs, both in terms of side effects and in the search for alternative treatments for anxiety.

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